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The proposed research was designed to utilize a combination of laboratory and field studies to identify physical, chemical, genetic, and physiological influences that govern the accumulation and biodegradation of polycyclic aromatic hydrocarbons (PAHs). These and related compounds are amoung the chemicals whose environmental fate has been targeted by the U.S. Air Force Bioenvironmental Research Program. We have conducted a prior, independent study that has shown that, despite the presence of PAH mineralizing micro-organisms, PAHs persist at a site where freshwater sediments are fed by PAH-contaminated groundwater. Hypotheses to be tested address fundamental mechanisms for the persistence of environmental pollutants, these include: (1) the rate of delivery meets or exceeds the rate of biodegradation; (2) the PAHs are not available to microbial populations due to sorption onto the sediment organic matter, complexation reactions with dissolved organic carbon, or due to the physical arrangement of the sediment matrix which prevents contact between PAHs and microorganisms; (3) the microorganisms may be physiologically limited by the presence of preferred metabolic substrates or toxic or inhibitory

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substances, or by the lack of proper final electron acceptors, electron donors, or inorganic or organic nutrients; (4) PAHs may persist simply due to restricted distribution and abundance of biodegradation genes in naturally occurring microbial populations. By working in an iterative manner between field observations and controlled laboratory determinations, we intend to systematically test the above hypotheses and thur identify constraints on microbiological processes that mineralize PAHs (naphthalene and phenanthrene) at the field site.

SECOND ANNUAL TECHNICAL REPORT
SUBMITTED TO
U.S. AIR FORCE OFFICE OF SCIENTIFIC RESEARCH

BIOENVIRONMENTAL HAZARDS PROGRAM
LIFE SCIENCES DIRECTORATE
BOLLING AFB DC, 20322-6448

(TO THE ATTENTION OF: **Dr. Walter J. Kozumbo**)

For AFOSR Project – 91-0436

GEOCHEMICAL, GENETIC, AND PHYSIOLOGICAL CONTROL OF POLLUTANT BIODEGRADATION

Second Project Period: 30 March 1993 - 29 March 1994

Date: April 29, 1994

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Panel C shows bacterial numbers from bulk sediment (single unreplicated sample) and sand sorbent (4 replicate samples) growth on phenanthrene.

Figure 29. Concentrations of coal tar-derived compounds retrieved from polyurethane foam sorbents inserted into the seep study area Fall 1993. Each point represents the average of 3 or 4 replicate samples. Compounds are analyzed by gas chromatography/mass spectrometry. Authentic standards were not available for all compounds therefore, absolute concentrations were not ascertained. Instead, the arbitrary units of chromatogram peak areas are displayed along the vertical axes.
Top = concentrations of naphthalene, 2 methylnaphthalene, 1 methyl, 2 cyclopropenyl, benzene, and 3 nitro-1,2dicarboxybenzene
Bottom = Concentrations of indene, 1,2,3-trimethylbenzene, 1,ethyl-2,methylbenzene, and 2,3-dihydro-4methyl-1H indene.

1.0 BACKGROUND

This document reports results of laboratory experiments carried out during the second fully funded year of a 3-year project entitled "Geochemical, Genetic, and Physiological Control of Pollutant Biodegradation" (a six month no cost extension period was sandwiched between years 1 and 2). For a detailed literature review, project description and statement of work, the reader is referred to the original proposal in its entirety.

1.1. Project Motivation and Goals:

A slightly modified version of the Abstract from the proposal that originally defined this project appears below. This synopsis is the most efficient way to familiarize the reader with project motivations and goals.

ABSTRACT

(from original proposal, 9/91)

The proposed research was designed to utilize a combination of laboratory and field studies to identify physical, chemical, genetic, and physiological influences that govern the accumulation and biodegradation of polycyclic aromatic hydrocarbons (PAHs). These and related compounds are among the chemicals whose environmental fate has been targeted by the U.S. Air Force Bioenvironmental Research Program. We have conducted a prior, independent study that has shown that, despite the presence of PAH mineralizing microorganisms, PAHs persist at a site where freshwater sediments are fed by PAH-contaminated groundwater. Hypotheses to be tested address fundamental mechanisms for the persistence of environmental pollutants, these include: (1) the rate of delivery meets or exceeds the rate of biodegradation; (2) the PAHs are not available to microbial populations due to sorption onto the sediment organic matter, complexation reactions with dissolved organic carbon, or due to the physical arrangement of the sediment matrix which prevents contact between PAHs and microorganisms; (3) the microorganisms may be physiologically limited by the presence of preferred metabolic substrates or toxic or inhibitory substances, or by the lack of proper final electron acceptors, electron donors, or inorganic or organic nutrients; and (4) PAHs may persist simply due to restricted distribution and abundance of biodegradation genes in naturally occurring microbial populations. By working in an iterative manner between field observations and controlled laboratory determinations, we intend to systematically test the above hypotheses and thus identify constraints on microbiological processes that mineralize PAHs (naphthalene and phenanthrene) at the field site.

1.2. Synopsis of progress from first 12-month period:

The Synopsis, below, is taken from p. 26 of the first Technical Report for this project (30 September 1991 – 29 September 1992). This Summary appears here in order to give the reader an appreciation of the significance and rationale for new experimental results reported in this volume.

Synopsis

This project has merged three research areas (field geochemistry, microbiology, and sorption chemistry) in order to understand the biogeochemistry of PAH compounds in a contaminated field site. Although one year of research effort has not explained why biodegradable PAHs anomalously persist at our field study site, substantial progress toward testing the relevant hypotheses has been made.

- Lack of naphthalene metabolism is caused neither by the absence of microbial metabolic capabilities, nutrient limitation, nor the presence of toxins in site samples.
- Sorption of PAHs has been found to affect their bioavailability, hence biodegradation, in complex and varying ways.
  - The notion that duration of sorption contact time completely governs PAH metabolism is simplistic. Under some circumstances mineralization of naphthalene does seem to be inversely proportional to sorption contact time. But this relationship is demonstrable with only certain microbiological populations. Thus, the idiosyncrasies and diversities of microbial metabolism are probably the key in understanding the sorption/bioavailability hypotheses. The size of the nonsorbed naphthalene pool also seems to be a significant influence.
  - When the Sorption/Bioavailability hypothesis was tested with phenanthrene, the type of sorbent emerged as a critical influence: regardless of inoculum source, no mineralization was observed when seep sediment (high in organic matter) was sorbent. In contrast, when sand was the sorbent (low in organic matter) mineralization did occur.
- Under anaerobic conditions favoring methanogens and sulfate reducers, no naphthalene metabolism occurred during a 16 day experiment. When oxygen and nitrate were supplied to the same sediments and microbial populations, rapid naphthalene mineralization occurred. Thus, simple oxygen and nitrate limitation has emerged as one of the most probable causes for PAH persistence at the field site. The presence of naphthalene mineralizing denitrifiers at this site, if confirmed, opens up a

broad area of physiological and genetic investigations comparing individual aerobic and denitrifying bacteria.

1.3 Synopsis of Progress from Second (6 month no-cost extension) Period

During the second period of the project (6-month no-cost extension of Year 1), weather precluded field work. Instead of visiting the site, measurements were performed on site-derived samples. Key areas of progress were:

- Isolation and characterization of a phenanthrene metabolizing bacterium whose behavior to sorbed phenanthrene mimics that of mixed populations in site-derived sediment samples.
- Discovery that mixed populations from the field-site sediments vary in their abilities towards metabolize sorbed phenanthrene, depending on sediment type and how the sediments were handled. Even phenanthrene sorbed to the organic-rich seep sediment can be metabolized. Thus, the role of physiological diversity in PAH metabolism has been confirmed and extended.
- Initiation of an approach to measure PAH metabolism by two independent analytical methods. This was designed to compare the fate of freshly-added and long-sorbed naphthalene. The insights promised by this type of experiment are very significant, but the details of PAH extraction and analysis procedures need to be improved before success is achieved.
- Establishment of serum-bottle-HPLC-based procedures for studying anaerobic metabolism of naphthalene. Results show that naphthalene mass balances can be assembled and that no denitrifying naphthalene metabolism could be detected. However, a clear oxygen limitation was demonstrated

2.0 PROCEDURES AND RESULTS

2.1 Overview

During the 12 month period that has passed since the last progress report, a variety of procedures for measuring microbial metabolism of PAHs, and for measuring PAH sorption reactions have been designed and implemented.

As is evident from the Table of Contents for this Report, progress in five areas have been made.

1. The field site-derived phenanthrene-metabolizing bacterium, *Sphingomonas paucimobilis* RSP1 (described briefly in the second report), has been further

characterized. The background physiological data presented here provide a sound foundation for future work aimed at understanding relationships between the fate of PAHs and the properties of this bacterium.

2. Investigations of microbial metabolism of sorbed PAHs have continued. These assays use a phenomenological approach which assess the responses of mixed cultures and pure cultures to PAHs aged for varying periods under aseptic conditions in the presence of sediments sterilized by γ-irradiation.

3. Data addressing the physiological, taxonomic, and molecular responses of soil and sediment microorganisms to naphthalene has been obtained. This component of the report focused on a population genetics approach to the distribution of naphthalene metabolism genes at the coal-tar contaminated study site. A diversity of isolates capable of metabolizing naphthalene have been isolated from 2 spatially distinct locations at the field site (the contaminated seep area and an uncontaminated adjacent hillside soil). The isolated bacteria have been characterized taxonomically (via a variety of procedures including the commercial BIOLOG series of substrate utilization tests) and genetically (by PCR amplifying and sequencing a portion of the *nah*Ac and *nah* R catabolic genes).

4. The mobility of PAHs and bacteria capable of PAH-metabolizing in site sediments has been investigated. These data were obtained in the Fall of 1993 from a field experiment in which an array of polyurethane foam plugs and small sterile sand bags were installed in the seep portion of the study site. Periodically, subsets of the originally clean sorbent materials were removed from the field site. Chemical [gas chromatograph/mass spectrometry (GC/MS)] analyses were conducted on material sorbed to the urethane foam and numbers of phenanthrene and naphthalene metabolizing bacteria adhering to the sand sorbents were also assayed.

5. Development of methods for extracting DNA from sediments. This topic is concerned with how to obtain DNA directly from environmental samples so that tools of molecular biology can be applied to microbial communities present in field sites. The results of this study are In press, due to appear in the May issue of Applied and Environmental Microbiology. The manuscript is attached to this report.

NOTE: In order to maintain continuity between reports, the numbering of Tables and Figures will continue consecutively with those in the first progress report.

2.2 Properties of *Sphingomonas paucimobilis* RSP1: a phenanthrene metabolizing bacterium

2.2.1. Taxonomic identification of strain RSP1

Strain RSP1 was identified by two commercially available bacterial identification kits: the API® Rapid NFT™ and the BIOLOG GN MicroPlate™. The Rapid NFT™ method identifies environmental isolates using a strip which includes tests for physiological capabilities and for carbon source utilization abilities. The BIOLOG™ system uses only carbon source utilization tests in a colorimetric 96-well plate assay. The results of these identification methods are shown in Table 11.

The API Rapid NFT™ kit clearly identified RSP1 as a *Sphingomonas paucimobilis* strain ("good identification"). The BIOLOG™ system, on the other hand, returned a "no identification" response when its computer database was searched because RSP1 did not achieve a similarity score of ≥ 0.5 (scale: 0.0 to 1.0) with any species in the database. However, a fairly high similarity score (0.43) was observed with *Sphingomonas paucimobilis* B, and the next closest match (*Pseudomonas vesicular*) identified with RSP1 with a similarity score of only 0.013. Furthermore, the fatty acid profile of RSP1 (data not shown) demonstrated the presence (most notably 14:0 2OH) and correct ratios of most of the fatty acids which distinguish *Sphingomonas paucimobilis* from other pseudomonads (Stead, 1992). In light of these results, strain RSP1 has been designated *Sphingomonas paucimobilis* RSP1.

TABLE 11. Identification of strain RSP1 by two commercially available phenotypic assays kits.

Commercial kit	Identification	Score[a]
API® Rapid NFT™	*Sphingomonas paucimobilis*	"good ID"
BIOLOG GN MicroPlate™	*Sphingomonas paucimobilis* B	0.43

[a] See text for explanation.

In addition to being fast and easy to use, the commercial identification kits which were employed have the advantage of providing useful physiological data about the organism tested. The detailed results of the tests from the Rapid NFT™

identification of RSP1 are reported in Appendix A. Carbon source utilization data obtained from the BIOLOG MicroPlate™ are reported in Appendix B.

2.2.2. General characterization

A summary of the general characteristics of RSP1 are listed in Table 12. RSP1 exhibits the characteristics of a typical pseudomonad; it is a catalase positive, oxidase positive, Gram negative rod. Electron microscopy of negatively stained cells of RSP1 shows that it is bipolarly flagellated (usually one to five flagella per pole). (Flagella can be visualized in the light microscope as well using fluorescent antibody techniques.) The electron micrograph also showed that RSP1 forms polyphosphate storage bodies when grown in PTYG5 medium; these are the dark intracellular structures. They are also known as "volutin granules", so named because these electron dense bodies appear to "volatilize" on the electron microscope screen when penetrated by the electron beam. The lighter stained structures within the cells may be polyhydroxyalkanoate (PHA) storage bodies.. However, when RSP1 cells were stained with a PHA specific dye, these structures were only detected only in long, filamentous RSP1 cells, which begin to appear late in the log phase of growth on PTYG5.

TABLE 12. Summary of general characteristics of strain RSP1.

Characterization test	Result
Gram reaction	-
catalase	+
oxidase	+
flagella	+[a]
polyphosphate bodies	+[b]
PHA bodies	+[b]
Growth curve in PTYG5	μ = 2.66 hrs
indole ---> indigo	+[c]
plasmid	-[d]
G+C content	71%[e]

[a] Flagellation is bipolar, usually one to five flagella per pole.

[b] Visible in electron microscope; PHA storage bodies were detected by specific staining only in the large, filamentous variants, which begin to appear late in the log phase of growth.

[c] Well characterized blue pigmentation change caused by aromatic dioxygenase enzyme.

[d] No plasmid has been found using three different methods.

[g] Based on sterilized thermal melting processes

A growth curve for RSP1 in PTYG5 has been prepared. Based on plate count results determined throughout the time course of the growth curve, RSP1 has a generation time of 2.66 hours (Table 12). Growth occurs slower in very rich medium (e.g., LB, TSA, full strength PTYG). Growth rates on aromatic substrates (PHN, NAH) in liquid culture is variable, but generally slow (estimated generation times between 2 and 15 days). It should be noted that other researchers have shown that the surface area of crystals of hydrophobic organic compounds limits the dissolution rate (i.e., availability) of the compound, which consequently limits growth rate. Thus, crystals were crushed as much as possible before addition to the MSB medium for growth of RSP1.

On most solid media, RSP1 colonies are yellow, circular, entire, convex, and smooth. On phenanthrene-supplemented minimal media (MSB-PHN) spray plates, colonies are relatively small and exhibit a rusty brown coloring, probably due to the accumulation of a metabolic intermediate in the phenanthrene degradative pathway. RSP1 does not have any known vitamin requirements, but MSB media was often supplemented with a vitamin solution to promote faster growth.

RSP1 was tested for its ability to convert indole to indigo. The ability to catalyze indigo formation from indole is a property of a nonspecific naphthalene dioxygenase enzyme. This reaction has been reported for other dioxygenases which were involved in aromatic hydrocarbon metabolism. The "indole to indigo" test became a presumptive diagnostic tool for designating metabolism of an aromatic hydrocarbon by a microorganism to follow a particular pathway which uses a dioxygenase versus a monooxygenase. More recently, however, the ability to convert indole to indigo has been reported for monooxygenases, as well. Nevertheless, since the oxygenases of some aromatic hydrocarbon-degrading microorganisms do not convert indole to indigo, this ability is significant in that it provides another level of characterization about a new organism.

The "indole to indigo" test was performed by placing indole crystals in the lids of inverted petri plates of RSP1 cultures which had been growing on phenanthrene (PHN) or naphthalene (NAP). The results are shown in Table 12. Indigo formation from indole by RSP1 grown on PHN or NAP was seen near the edges of the culture streaks, where growth and metabolism were probably the

most active. When grown on NAP, PpG7, the positive control strain which produces a naphthalene dioxygenase, showed strong indigo formation within hours of exposure to indole. The negative control strain, *E. coli* B/r, which was grown on LB medium (it cannot grow on PAHs), showed no indigo formation from indole.

Because the ability to utilize aromatic hydrocarbons is often encoded on large catabolic plasmids, three plasmid isolation procedures were attempted in an effort to determine if RSP1 harbors such a plasmid. Only the method of Anderson and McKay (1981) produced a strong plasmid band when performed on the positive control strain, *Pseudomonas putida*, PpG7, which harbors the 83 kb NAH7 plasmid encoding NAP degradation. No plasmid was detected in strain RSP1 by this method. That RSP1 and PpG7 are in the same family of microorganisms (Pseudomonaceae) and that chromosomal DNA was extracted from both of these organisms by this method lends support to the fact that any plasmids harbored by RSP1 should have been detected. The possibility that there is not enough plasmid DNA to be seen may be excluded because equal volumes of approximately equal density cultures of RSP1 and PpG7 were used, and the NAH7 plasmid of PpG7, which, like most large catabolic plasmids, is a low copy number plasmid, was easily detectable. Furthermore, a portion of the RSP1 culture used for the plasmid isolation procedure was plated onto the general heterotrophic medium, PTYG5, immediately before the isolation procedure was started. A full 100% (17/17) of the RSP1 colonies which were picked and streaked onto MSB-PHN and MSB-NAP media showed growth on each of these media. Thus, RSP1 was not cured of the plasmid during growth on PTYG5 for the isolation procedure. Despite these arguments, however, it can only be said that no plasmid has been found in RSP1 by the methods employed.

Total DNA was isolated and purified from RSP1. The G+C content was calculated to be approximately 71% based on the thermal melting curve (Table 12). This estimate may be high, however, because the *E. coli* standard DNA, which is known to be about 51% G+C, was calculated to be about 58% based on data from the same thermal vamp. In any case, it is safe to say that RSP1 is GC rich organism, with a G+C content probably between 65 and 75%. This is important to note for possible future experiments with this organism, which might include DNA sequencing and DNA hybridization studies/gene probing.

2.3. Continued Investigations of Microbial Metabolism of Sorbed PAHs

As discussed extensively in the first two reports from this project, one hypothesis, for explaining the presence of PAH compounds at our study site is reduced bioavailability through sorption reactions. The experiments described below continue a series of tests designed to examine if the extent of microbial metabolism of naphthalene and phenanthrene may be inversely proportional to the duration of contact time between these PAHs and sediment sorbents.

Aging experiment #6: Phenanthrene added to seep, and sand sediments inoculated with enriched pure cultures or unenriched sediments.

Procedures. Essentially the same procedures were followed for Experiments # 4 (Fig. 9 of 9/91-9/92 AFOSR report) and 5 (Fig. 17 of 9/92-3/93 of AFOSR report), except only two aging periods were examined (0 and 28 days). Key variables were the inocula (a mixture of 2 phenanthrene-degrading bacterial cultures or sand or seep sediments). Also, special care was taken to be sure that all treatments had the same ratios of solids to liquids. This is a challenging objective when inocula initially have very different water contents; but the solids/liquid ratio is particularly important in efforts examining the response and bacteria to sorption/desorption reactions.

Results. Results of the sixth aging experiment are shown in Fig. 23A-C. Panel A shows the response of the seep sediment inoculum to aged and freshly added ^{14}C-phenanthrene sorbed to low organic-matter sand from the source area and high organic-matter seep sediment. As was found in aging Experiments #4 (9/91-9/92 AFOSR report), and #5 (9/92-3/93 AFOSR report), the seep inoculum was unable to metabolize phenanthrene sorbed to γ-irradiated seep material. With sand as sorbent however, the phenanthrene was metabolized. Furthermore, this metabolism was greatest for freshly added phenanthrene and least for the aged phenanthrene/sand mixture. Thus, when the inoculum was seep sediment that was not intentionally enriched on aqueous phase phenanthrene, the organisms found aged phenanthrene to be less available than unaged. This suggests that enrichment on aqueous phase PAH may not always be the key feature in understanding the effect of aging on PAH metabolism. Alternatively, many seep sediments may be so wet as to be physiologically predisposed towards utilization of aqueous-phase PAHs.

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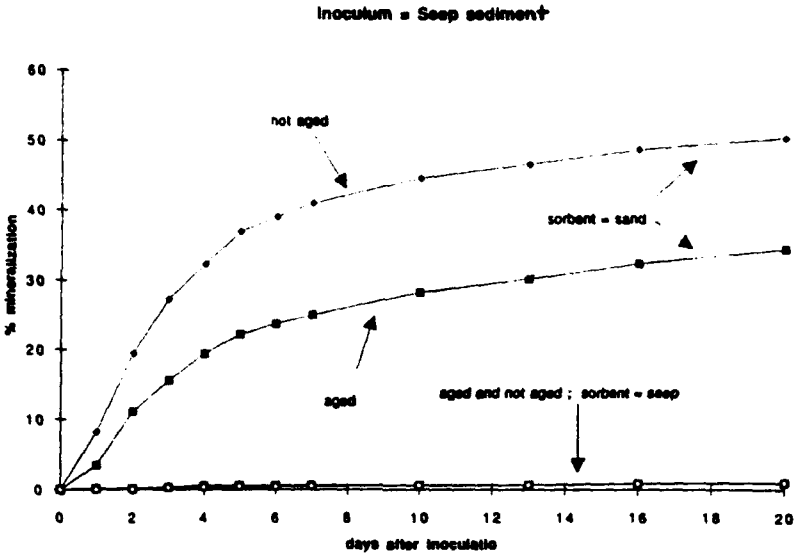

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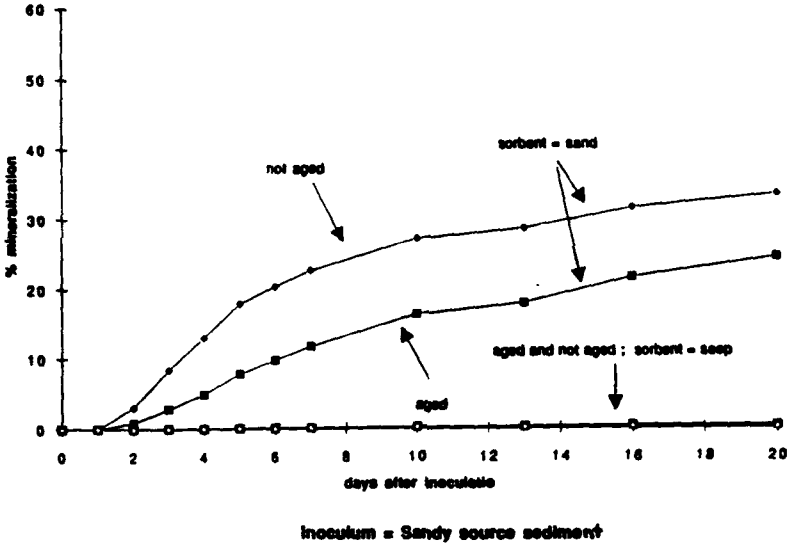

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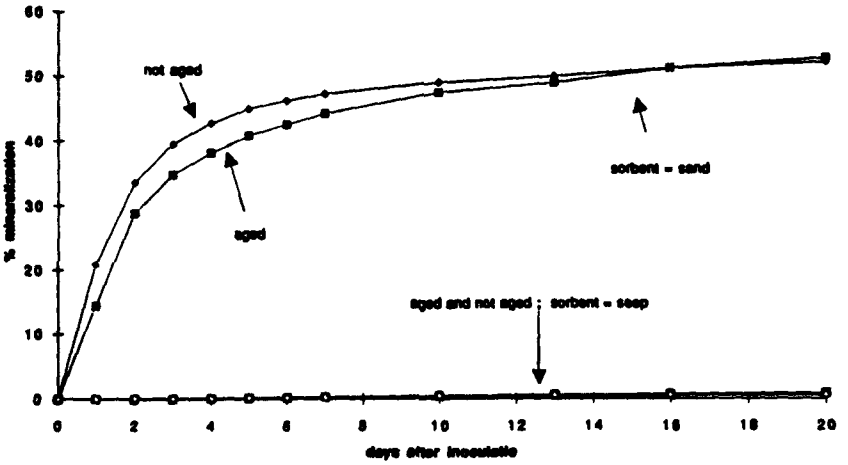

FIGURE 23. Mineralization of phenanthrene aseptically aged with sand and seep sediments as sorbent and subsequently inoculated with either unenriched seep sediment (A) or *Sphingomonas paucimobilis* RSP1 (B) or unenriched sandy source sediment (C).

Panel B of Fig. 23 shows the response of a mix of pure cultures phenanthrene metabolizing bacteria, *S. paucimobilis* RSP1 and strain RS2A, to ^{14}C phenanthrene as presented in aged and unaged forms sorbed to sand and seep materials (as described for panel A). The results are again similar to those found in Experiments #4, 5 (as previously referenced), and 23A: (i) when the seep sediment was sorbent, no phenanthrene mineralization was observed regardless of aging; (ii) when sand was sorbent, the phenanthrene was mineralized — at the highest rate and greatest extent for unaged phenanthrene (see especially, results from Experiment #4 in previous report). T results from the mix of pure cultures (Panel B of Fig. 23) very closely matched . of the seep inoculum (panel A of Fig. 23). This fact is encouraging because it provides impetus for further exploring the physiology and biochemistry of sorbed substrate utilization by studying the effect using pure cultures. The agreement between panels A and B also suggests that the seep-sediment inoculum was, in fact, pre-adapted toward aqueous phase phenanthrene because pure cultures (Panel B) had been grown on dissolved phenanthrene prior to initiating mineralization tests.

Panel C of Fig. 23 shows the response of unenriched sandy source inoculum to ^{14}C phenanthrene as presented in aged and unaged forms sorbed to sand and seep materials. The results are similar to those of panels A and B, and data from previous experiments #4 and #5, but there are also some important differences.

(i) Unlike results in Fig. 17 (Expt. #5), but identical to Fig. 9 (Expt. #4), the sand inoculum failed to metabolize phenanthrene sorbed to the seep sediment. This inconsistency is difficult to understand but may be attributed to either spatial/temporal variation in the inoculum itself or, perhaps more likely, to the difference in amount of sand inoculum added to experiments reported in Figs. 17 and 23. In the former, the large inoculum/sorbent ratio (1:2.5) may have allowed re-equilibration of seep-sorbed phenanthrene from "unavailable" sites on the organic-rich seep to "available' sites on the sand. This shift may not have been possible in the experiment contributing to Fig 6 in which the inoculum to sorbent ratio was (1:6).

(ii) When the sand was sorbent, phenanthrene was metabolized at virtually identical rates and extents regardless of the aging treatment. To interpret why the sand-derived inoculum utilized the aged and unaged sand-sorbed phenanthrene equally well, requires postulating properties about the inoculum which are uncertain. In comparing panels A and C of Fig. 23 for instance, it is clear that the seep inoculum and sand inoculum responded differently to the aged materials. The explanation may rest in particular microorganisms present in one sediment but not the other or their physiological

predisposition to aqueous vs. sorbed PAHs.... but presently, we cannot distinguish between these or any other possible explanations.

Aging Experiment #7: An examination of diauxy, toxicity, and sorptive properties induced by γ-irradiation.

Procedures. Several different approaches to mineralization experiments were carried out to further explore lack of phenanthrene metabolism that has been documented above when γ-irradiated seep sediments were used as sorbent. The idea was to investigate whether or not this lack of metabolism was due to (a) a diauxic effect: production of readily utilizable organic compounds (through γ-irradiation) that may be preferentially metabolized by the inocula (Fig. 24A); (b) a toxicity effect; production of toxic compounds that may prevent metabolism (Fig. 24B); (c) some other γ-irradiation-induced property of the seep; (Figs. 24C, D).

Results. To investigate a possible diauxic effect in γ-irradiated seep sediments, ^{14}C phenanthrene was added to fresh seep sediment with and without addition of 10 ppm glucose (a strong inducer of catabolite repression, hence diauxy). Cumulative $^{14}CO_2$ production (panel A) showed that the glucose had no effect. Thus, we conclude that lack of phenanthrene metabolism in the presence of γ-irradiated seep was not due to diauxy.

To examine possible toxin production by γ-irradiation, we used strain RSP1 in a bioassay in which $^{14}CO_2$ production from ^{14}C-glucose was monitored after the cells were added to γ-irradiated seep sediment. As is apparent in Panel B of Fig. 24, there was not even a lag in glucose mineralization, which was quite extensive. Thus, the γ-irradiated seep did not contain general metabolic inhibitors.

To reaffirm that the protection of phenanthrene (lack of mineralization) observed when γ-irradiated seep was used as sorbent (see Figs. in earlier reports), we monitored $^{14}CO_2$ produced from ^{14} C-phenanthrene added to 3.5 g fresh, nonsterile seep material and to 1.5 γ-irradiated sorbent inoculated with 0.5 g of fresh nonsterile seep sediment. Results of $^{14}CO_2$ produced by these treatments appear in Panel C of Fig. 24. The non γ-irradiated seep sediment rapidly mineralized the ^{14} C phenanthrene; however, the γ-irradiated material failed to do so. In light of results shown in Panels A and B of Fig. 24, this lack of phenanthrene metabolism when in contact with γ-irradiated seep, must be attributed to sorption reactions. The weakness of studying this phenomenon, however, is

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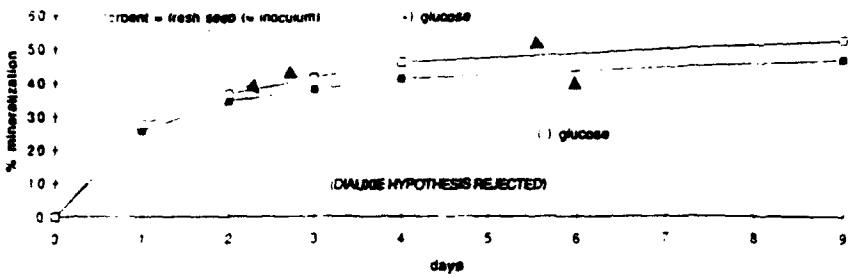

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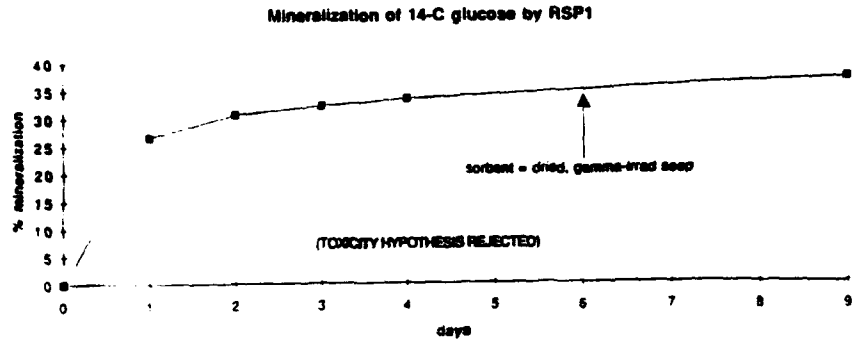

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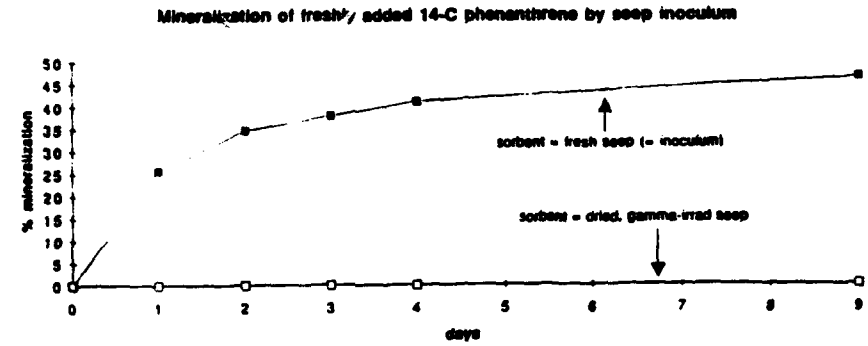

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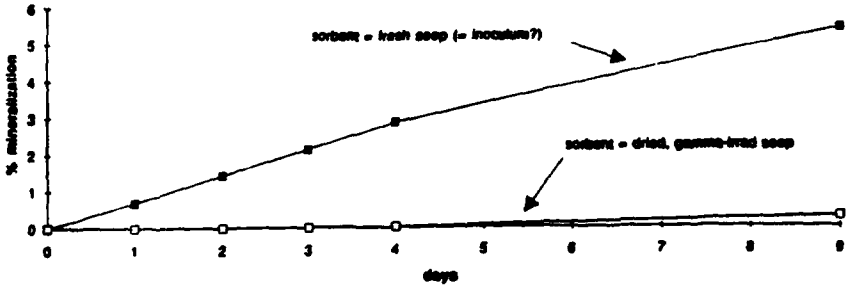

FIGURE 24. An examination of diauxie, toxicity, and sorbtion effects upon microbial mineralization activity Panel A reports $^{14}CO_2$ production from radiolabeled phenanthrene in the presence and absence of 10 ppm glucose. Panel b reports glucose mineralization by strain RSP1 in the presence of γ-irradiated seep sediment. Panel C shows $^{14}CO_2$ production from radiolabeled phenanthrene added to fresh and γ-irradiated seep sediment. Panel D follows $^{14}CO_2$ production from radiolabeled phenanthrene added to γ-irradiated seep with and without additional fresh seep material and inoculated with strains RSP1.

its possible irrelevance to our field site(sediments there are not γ-irradiated). To further examine this phenomenon of phenanthrene being protected from microbial attack, we postulated that a mixture of γ-irradiated sediment would lead to inhibition of mineralization. In panel D of Fig. 24, $^{14}CO_2$ production from ^{14}C-phenanthrene is reported from a mixture of 3 g fresh seep material (plus accompanying microorganisms) with 0.25 g of γ-irradiated sterile seep, and with an additional inoculum of strain RSP1. As a control in this experiment, the RSP1 inoculum was also added to ^{14}C phenanthrene mixed with a sorbent consisting entirely of γ-irradiated seep. Data in Panel D of Fig. 24 show again that phenanthrene is rendered unavailable to RSP1 when the sorbent was γ-irradiated seep. Furthermore, with a ratio of 0.25 g γ-irradiated seep to 3 g non irradiated seep, mineralization occurred — but to a far lesser degree (see vertical scale of % mineralization axis) than would be expected for unimpaired phenanthrene metabolism by RSP1 (see Figs. 17 and 23).

Summary of Aging/Bioavailability Experiments.

The impetus for attempting to understand relationships between sorption reactions and PAH mineralization has been from two related issues. First, because we are concerned with the possibility of exploiting microbial processes for eliminating contaminants from soils, sediments, and ground water, we need tools for predicting the extent of PAH metabolism in contexts where sorption occurs. We need to know the degree to which sorption reactions govern biological reactions. The second motivation is derived from a need to understand our own assay procedures. Wherever ^{14}C-labeled compounds are used in our laboratory experiments, these compounds are necessarily "freshly added". In interpreting the results of such tests, we need to know if the data can be extrapolated to the entire pool of contaminants (both recently sorbed and long sorbed).

Have data from Experiments 1–7, as summarized here and in Table 13, answered the above questions? Alas, we cautiously reply "not yet", because rather than black and white results, we have discovered areas of gray. Key points follow:

(1) Without a doubt, sorption reactions affect microbial metabolism of PAHs. For instance, in Experiment #3, when the only variable distinguishing different treatments was duration of contact between naphthalene and both sand and seep sorbents, the extent of naphthalene mineralization was related inversely to that contact time.

TABLE 13. SUMMARY OF METHODS DEVELOPMENT AND RESULTS OF AGING/BIODEGRADATION EXPERIMENTS[A]

		Methodological Details						
Expt.	Chemical	Principal Variables	Aging vessel and concentration	Inoculum	Mineralization vessels and ratio of wet sediment to other fluid in slurry	Comments	Support for Aging/Bioavailability Hypothesis	Conclusion
#1	Nap	aging period = 0-20 d sediment = seep	Ichem vial, 15 ppm nap	5 g seep added to 100 ml minimal medium saturated with naphthalene shaken for 2 days (Enriched)	serum bottles x butyl septa, 2 ml of inoculum added to 8 g wet seep sediment	1a) During aging, mixing may not have been sufficient to attain homogenous distribution of ^{14}C. Also, ^{14}C may have escaped. 1b) During mineralization, stopper and CO_2 trap may have sorbed the naphthalene. 1c) ^{14}C naphthalene may have volatilized from sediments during preparation for the mineralization assay.	? ? ?	Need to improve mixing, improve mineralization assay and minimize naphthalene volatilization losses. Poor methods. Slight support of/biomineralization hypothesis (Fig. 5 of 1-6/92 report)
#2	Nap	aging period = 0-21 d sediment = sand and seep	glass ampoule, 3 ppm nap	1 g seep to 100 ml 0.005M $CaSO_4$ (No enrichment)	Pierce vial/Teflon septa, 1 ml of inoculum added to 5 g wet seep sediment	2a) point 1c above not solved 2b) During mineralization assays the septa may leak after being punctured.	-	Despite methodological improvements, results contrasted strikingly with those of experiment #1. No relationship between aging and rate or extent of mineralization (Fig. 6 of 1-6/92 report)
#3	Nap	aging period = 0-28 d sediment = sand and seep	glass ampoule, 3 ppm nap	2 g seep added to 150 ml minimal medium saturated with naphthalene shaken overnight (Enriched)	Pierce vial/Teflon septa, 0.5 ml of inoculum added to 4 g wet sediment	3a) See 2a 3b) see 2b 3c) Dissolved naphthalene may have accompanied the inoculum	+	With more refined procedures, confidence in results grows. Trend suggests that mineralization is governed by contact time, but the inoculum may also be critical. (Fig. 7 of 1-6/92 report)

Table 13 Continued

Expt.	Chemical	Principal Variables	Aging vessel and concentration	Inoculum	Mineralization vessels and ratio of wet sediment to other fluid in slurry	Comments	Support for Aging/Bio-availability Hypothesis	Conclusion
#4	Phen	aging period = 0-20 d sorbent = sand and seep	glass ampoule, 1.5 ppm phen	2 ml of enrichment culture from either seep or sand. Medium contained phenanthrene yeast extract, peptone, and glucose	Pierce vial/Teflon septa, 2 ml of inoculum to 3 g (sand) or 4 g (seep)	4 a) see 2a, phenanthrene is, however, less volatile b) see 2b c) see 3c	+ –	See #3 Little aging effect, Seep inoculum supports Aging/Bioavailability hypothesis (Fig. 9) Seep protects phenanthrene from mineralization inocula (Fig. 4, this report)
#5	Phen	aging period = 0 and 96 days Sorbent = sand, seep inoculum = sand, seep, or strain RSP1	glass ampoule, 1.5 ppm	Sediment inoculum unenriched Cultures grown on phenanthrene plates, then suspended in phosphate buffer	Pierce vial, Teflon Septa 1 g sediment or 0.5 ml phen grown cultures to 2.5 g sediment.	(See 4 a, b, c) 5 a) Only duplicate mineralization vials 5b) Ratio of liquid to solvent not consistent 5c) Sediment inoculum not mixed with sorbent 5d) Large sediment inoculum may have shifted sorption-equilibrium off of the original sorbent	+ –	Of all inocula tested, only the sand was capable of mineralization. RSP1 and seep inocula support sorption/Bioavailability Hypothesis, sand inoculum does not (Fig. 5, this report)
#6	Phen	aging period - 0 and 28 days sorbent = sand seep inocula = sand, seep, RSP1, RSA2	glass ampoule, 1.5 ppm phen	RSP1 and RSA2 grown in liquid medium on phenanthrene unenriched seep and source sediment	Pierce vials 0.5 ml culture or 0.5 g sediment to 3 g sorbent	(See 4 a, b, c) 6a) RSP1 and RSA2 inocula were combined 6b) Sorbent and inoculum were well mixed 6c) All liquid to solid ratios were held constant	+ –	γ- irradiated seep still protect regardless of inoculum Aging effect in the seep, pure culture, but not sand inocula. (Fig. 6, this report)

Table 13 Continued

Expt.	Chemical	Principal Variables	Aging vessel and concentration	Inoculum	Mineralization vessels and ratio of wet sediment to other fluid in slurry	Comments	Support for Aging/Bio-availability Hypothesis	Conclusion
#7	Phen and Glu	diauxy toxicity γ-irradiation	no aging examined here 1.5 ppm phenanthrene	RSP1 grown on liquid glucose medium Unenriched sediment	Pierce vial and teflon septa 0.5 ml liquid to 3.5 g sediment or 0.5 g sediment to 1.5 g γ-irradiated	7a) Sorbent and inoculum were well mixed 7b) All liquid to solid ratios were held constant 7c) All tests were well mixed	+	– Fresh seep metabolizes phen, fresh seep inoculated into γ-irradiated sediment does not – RSP1 metabolizes glucose in the presence of γ-irradiated sediment – seep microorganisms metabolize phen ± glucose – A mixture of seep organisms and RSP1 show reduced phenanthrene metabolism when γ-irradiated seep is added – RSP1 fails to metabolize phen from γ-irradiated seep. (Fig. 7, this report)

[A] Please note, Experiments #1-3 appeared in Table 2 of the 1/1/–6/30/92 progress report for this project.

Nap = Naphthalene

Phen = Phenanthrene

Glu = Glucose

Likewise, in Experiments #4, 5, 6, and 7, complete inhibition of phenanthrene metabolism was seen when the sorbent was γ-irradiated seep. We concluded that this occurred as a result of sorption reactions by testing the alternative hypotheses of diauxy and toxicity.

Furthermore, in all of the aging/bioavailability experiments examining phenanthrene metabolism, the aged materials inoculated with pure cultures and seep inocula metabolized aged phenanthrene to a lesser degree than the freshly-added substrate. Exceptions both to protection by the seep (as sorbent) and to reduced metabolism of aged material, were consistently found with inocula derived from sandy source sediments.

(2) Despite the qualitative findings in point 1 (above), explanations providing predictive power about sorption and PAH metabolism have been evasive. Reasons for our inability to find definitive answers probably lie in limitations of our methodologies. Field samples from Site 24 sediments must be used in our studies to guarantee relevance; however, the samples are heterogeneous and contain unknown mixtures of microbial populations. Unifying concepts such as "enrichment for aqueous phase PAHs should produce populations unable to metabolize sorbed PAHs" make sense in "explaining" why aging had a strong effect on naphthalene metabolism in Experiments #3 and #2. This enrichment effect also helped to "explain" metabolism of phenanthrene by seep sediments in Experiment #4. However, when phenanthrene metabolism was further scrutinized in later experiments, the concept broke down. In Experiments #5 and #6, sediment inocula were "unenriched" (i.e., added to the mineralization vials without prior addition of water and phenanthrene in a way that might favor metabolism of aqueous-phase phenanthrene). Therefore, these unenriched sediments should have been indifferent to sorbed vs. aqueous-phase phenanthrene. But instead, the aging effect was seen for the seep inoculum, but not the sand inoculum. We can only explain this by evoking differences in the microbial communities of the two inocula and uncertainties about our ability to presume anything about how to assure a microbial community's predisposition toward sorbed PAHs. The beauty of utilizing pure cultures is an ability to control their previous history. Thus, with cultures grown on dissolved-phase phenanthrene, (Experiments #5, and #6), an aging effect was consistently seen. This is encouraging.

(3) In recognition of the importance of methods, it may be prudent to point out that γ-irradiation of sediments, performed to eliminate background microbial populations, was only one of several possible strategies to examine the Aging/Bioavailability Hypothesis. We chose this approach for good reasons. However, the finding that ^{14}C-phenanthrene is available for metabolism when added to nonsterile seep, but not γ-irradiated seep is

disconcerting, because it implies that the information gathered suffered from experimental artifacts. This is true, but the information obtained still provides insights into sorption/bioavailability mechanisms. Should this project be renewed in the future, other (non γ-irradiation) approaches for examining aging/biodegradation relationships can be explored and contrasted with the data presented here.

2.4. Physiological Taxonomic, and Molecular Responses of Soil and Sediment Microbial Communities to Coal-Tar Contaminants

Introduction

The response of naturally occurring microbial communities at our coal-tar-contaminated field site was explored using a variety of field, microbiological, and molecular methods. The overall scope for this portion of the investigation is as follows:

1. Based on proximity to the groundwater contaminant plume we identified soil and sediment microbial communities that either were or were not exposed to high naphthalene concentrations hence were not adapted to metabolize naphthalene.
2. That degree of adaptation was measured (and defined) using lag time prior to onset of $^{14}CO_2$ production by soil or sediment samples amended with ^{14}C-naphthalene. The locations identified for in-depth study were seep sediments (adapted) and an adjacent hillside soil (unadapted).
3. Naphthalene degrading bacterial isolates were obtained from both seep and hillside samples in a manner designed to reflect *in situ* proportions of the isolated members of microbial community. A total of 41 isolates were obtained, 19 from the seep and 22 from the hillside.
4. In addition to showing that the isolated bacteria grew on naphthalene (i.e., produced $^{14}CO_2$ from ^{14}naphthalene and produced large colonies on agar media when naphthalene was provided as a carbon source), each was characterized and classified using standard microbiological procedures (Gram stain, catalase test, oxidase test) and commercial bacterial identification kits (BIOLOG and API-NFT).
5. Isolates are being characterized using molecular procedures, as well. Methods utilized include using the polymerase chain reaction (PCR) to amplify *nah*Ac (the gene encoding naphthalene dioxygenase, the first enzyme in naphthalene catabolism) and *nah*R (encoding the positive regulatory protein), dot-blot hybridization with a *nah*Ac gene probe, and sequencing of *nah*Ac.

6. Implementing this overall strategy will constitute a Ph.D. thesis, being completed by James B. Herrick. The principal goal of the thesis is to gain new insights into the response of naturally occurring microbial communities to PAH contaminants can be obtained using rigorous application of a variety of conventional and molecular procedures.

Methods

Sample selection and characterization. Sampling was done at two sites in the seep area. One sample was taken aseptically from saturated sediments approximately 10 cm below the surface and immediately within the contaminated portion of the seep. The other was taken from moist soil at approximately 2 cm depth and approximately one meter from the first and uphill in a direction opposite the contaminated plume source. Soil and sediment samples were tested for mineralization of ^{14}C-labeled naphthalene as previously described. Preparation and counting of total bacteria by fluorescent direct counting were also done routinely in our laboratory. For dilution plating, 10 grams of each sample were suspended in 90 ml. of sodium pyrophosphate, further diluted in potassium phosphate buffer and plated in three replicates per dilution on Difco R2A medium (for total counts) and on Stanier's Mineral Salts B (MSB) medium (for naphthalene bacteria counts and isolation). Naphthalene was supplied as vapor. Viable counts were determined and colonies sampled for isolation after 72 hours growth at 22° C. Samples were tested for ^{14}C-naphthalene mineralization, fixed for microscopic direct counts, and dilution-plated within 24 hours of sampling.

Strain isolation and characterization. For each sample, 30 colonies greater then 1 mm in diameter were sampled randomly from one replicate plate having between 30 and 200 well-defined colonies. These were purified on MSB plates + naphthalene vapor and checked for purity on complete medium (5% PTYG) plates. Presumptive growth on naphthalene was determined by plating on MSB with and without naphthalene vapor. Metabolism of naphthalene was confirmed by assaying ^{14}C-naphthalene mineralization in sealed flasks. Gram stain and KOH, catalase, and oxidase tests were carried out on each isolate using standard methods. Two kits for bacterial identification, API-NFT and BIOLOG, were employed according to the manufacturers' instructions. Cluster analysis was performed using the BIOLOG program MLClust. Conversion of indole to indigo, an insoluble blue dye, was used as a presumptive assay of dioxygenase activity. Strains were grown on MSB + naphthalene at room temperature for six days, then incubated in the presence of indole, supplied as vapor. Any blue color in colonies after 9 1/2 hours was scored as positive. Results were identical after 24 hours.

DNA hybridization. Digoxigenin labeling of the *nah*Ac probe was carried out as has been previously described [Herrick, *et al.*, 1993 Appl. Environ. Microbiol. 59:687-694]. Total genomic DNA was extracted from pure cultures using standard methods. 5 μg DNA from each isolate was denatured in 0.1 volume of 4M NaOH/0.1 M Sodium EDTA. It was then dot-blotted onto an MSI Magna Graph nylon membrane and oven-baked according to the manufacturer's instructions. Prehybridization and hybridization conditions were as described by Boehringer-Mannheim. Blots were washed 2 times, 5 minutes each at room temperature in 2X wash buffer (2X SSC + 0.1% SDS) and again 2 times, 15 minutes each at 60° C in 0.5X wash buffer (0.5X SSC + 0.1% SDS: ca. 25% mismatch), at 65° in 0.25X wash buffer (ca. 15% mismatch), or at 65° in 0.1X wash buffer (ca. 8% mismatch). Percent mismatch was calculated using the method of Meinkoth and Wahl. Bound probe was detected by chemiluminescent exposure of X-ray film according to the manufacturer's instructions.

Results

Sample characterization and mineralization

Two samples were collected for comparative studies on naphthalene degrading bacterial guilds, one from the contaminated seep itself and one from surface soil approximately one meter distant and slightly uphill in a direction opposite the contaminated plume source. Each sample was tested for the ability of its microflora to mineralize ^{14}C-labeled naphthalene in sealed flasks. As was noted in previous studies in our laboratory, the contaminated seep sediment rapidly mineralized naphthalene without a lag phase (Fig. 25). Mineralization by the hillside soil, on the other hand, exhibited a very slow increase over the course of the experiment. Very similarly-shaped curves were also observed for two other samples taken from presumably uncontaminated seep sediments near the contaminated seep (data not shown). Mineralization curves for ^{14}C-labeled p-hydroxybenzoate on the other hand, a compound easily metabolizable by many heterotrophic bacteria, were equivalent for all samples (not shown). Also, total counts and viable counts of bacteria from the samples were equivalent (Table 14). Thus, differences in mineralization observed for previously-exposed and -unexposed samples were not due to differences in total or total viable bacteria, nor to differences in aromatic metabolic activity. Both previously exposed and unexposed samples harbor native naphthalene-degrading microbes. Naphthalene guilds from contaminated and uncontaminated samples show very different responses to added naphthalene, however, indicating differences in the degree and possibly the manner of previous adaptation to the compound.

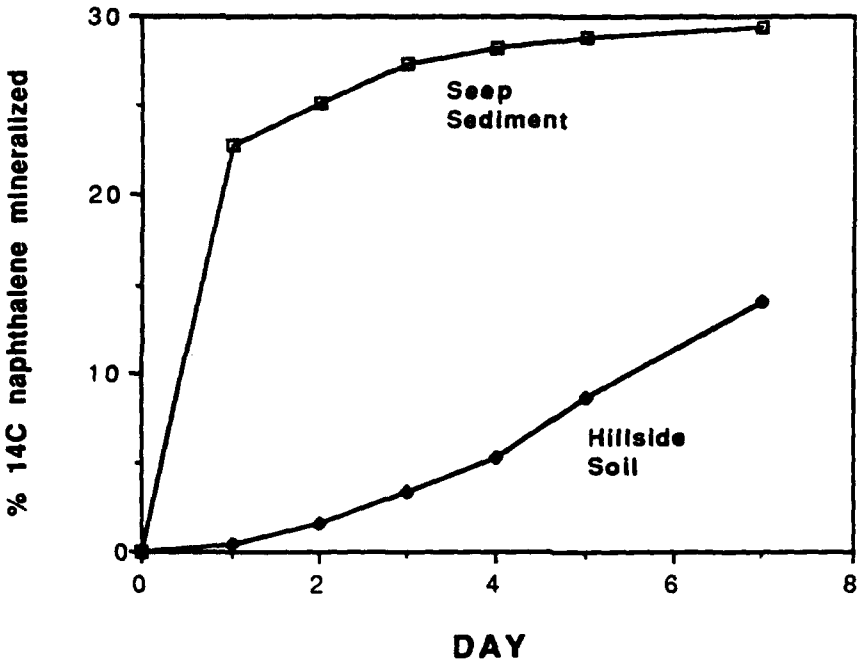

Figure 25. Naphthalene mineralization by seep and hillside samples.

TABLE 14. BACTERIAL COUNTS OF SAMPLES USED IN THIS STUDY.

Sample Designation	AODC (· gdw $^{-1}$)[a]	Total CFU (± SD) · gdw $^{-1}$
Seep sediment	$4.98 \cdot 10^9$	$1.6 (\pm 0.3) \cdot 10^6$
Hillside soil	$3.37 \cdot 10^9$	$1.7 (\pm 0.5) \cdot 10^6$

[a]Mean of two smears, 15 fields counted per smear.

Characterization and taxonomic structure of naphthalene-degrading guilds.

In order to further examine, on the organismal and genetic levels, the naphthalene-degrading bacterial guilds at the study site, individual bacteria were randomly isolated and purified from the seep and hillside samples. Samples were diluted and plated directly onto minimal medium under naphthalene vapor in an attempt to avoid bias due to prior enrichment and thus to identify the numerically dominant culturable naphthalene-degrading bacteria. Twenty purified isolates from the contaminated seep and 23 from the hillside soil were chosen based solely on differences in growth on plates with and

without naphthalene vapor. Of these, 19 seep and 22 hillside isolates were able to fully mineralize ^{14}C-naphthalene to $^{14}CO_2$ in sealed flasks after three days. These isolates were subsequently characterized and identified using standard microbiological methods and the test kits API-NFT and BIOLOG (Table 15).

Although the API test provides useful additional metabolic information, such as isolates' capability to respire using nitrate and nitrite or to ferment glucose, it can be seen from Table 15 that the BIOLOG system was far more successful in identifying isolates. Identified isolates from the hillside sample were primarily Gram-negative, oxidase negative rods of the species *Burkholderia* (formerly *Pseudomonas*) *gladioli* and the related species *Pseudomonas glathei*. These species are known primarily as plant pathogens and have not, to our knowledge, previously been identified as degraders of polycyclic aromatic hydrocarbons. They are members of the β-proteobacteria, a group widely-divergent from the γ-proteobacteria containing the type naphthalene-degrading strain *Pseudomonas putida* G7 and the other well-known ("true" or Type I *Pseudomonas*) naphthalene-degrading taxa. Type I pseudomonads, notably *P. putida* and *P. fluorescens*, were heavily represented among the seep isolates, however, as well as β-proteobacteria such as *A. faecalis* and *S. mizutaii*. Notable, also, is the presence among the seep isolates of the Gram-positive *Micrococcus diversus*.

The contaminated seep isolates represent a taxonomically more diverse group than do the uncontaminated hillside isolates. This is represented graphically in Figure 26. Cluster analysis based on BIOLOG carbon source utilization by each isolate shows that, within the seep isolates, there are at least four major groupings, one representing the classical γ-proteobacterial Type I *P. putida* and *P. fluorescens* species, another solely by *P. corrugata* Cg7, a third by the β-proteobacterium *A. faecalis* Cg11 and its possible relative Cg4, and the fourth grouping by the Gram-positive *M. diversus* Cg3 and Cg18. Another group, not shown on the figure, is represented by the newly-identified *Sphingobacterium mizutaii* Cg21, a member of the α-proteobacteria.

Exploring the genetic basis of naphthalene mineralization

When the isolates shown in Fig. 26 were examined for PCR-detectable genes in naphthalene metabolism (*nah R and* nahAc), none of the isolates contained the regulatory gene (*nah*R) (Fig. 27). Furthermore, none of the hillside isolates contained the structural gene (*nah*A) (Fig. 27).

The gene distribution data in Fig. 27 represents small scale genetic biogeography. A selective pressure at the field site (coal tar contamination) has altered the native microbial community in a variety of ways. By using the PCR to attempt to amplify 2 genes known

TABLE 15. CHARACTERIZATION AND IDENTIFICATION OF NAPHTHALENE-MINERALIZING BACTERIAL ISOLATES

Number	Source	Gram Stain/ Morphology	Oxidase	API-NFT I.D. (closest taxon)[a]	% id[b]	T index[c]	BIOLOG I.D.
Cg1	Seep	neg. rod	+	*Ps. putida*	99.7	0.98	*Pseudomonas putida* 0.79
Cg2	Seep	neg. rod	+	*Pseudomonas*. sp. (aureofaciens)	(82.2)	(0.85)	*Ps. fluorescens* 0.67
Cg3	Seep	pos. rod	-	ND[d]	ND	ND	*Micrococcus diversus* 0.739
Cg4	Seep	neg. rod	+	No I.D.	ND	ND	No I.D. (*Ps. fluorescens* E 0.41)
Cg5	Seep	neg. rod	+	*Pseud.* sp. (*fluorescens*)	(70.4)	(T=0.79)	*Ps. fluorescens* B 0.51
Cg6	Seep	neg. rod	-	ND	ND		No I.D.
Cg7	Seep	neg. rod	+	No I.D.			*Ps. corrugata* 0.51
Cg8	Seep	neg. rod	+	No I.D.			*Ps. fluorescens* B 0.50
Cg9	Seep	neg. rod	+	No I.D.			*Ps. fluorescens* B 0.67
Cg11	Seep	neg. rod	+	*Ps. picketii*			*Alcaligenes faecalis* 0.53
Cg12	Seep	neg. rod	+	No I.D.			*Pseud.* sp. (*putida* B 0.46)
Cg13	Seep	pos. rod	-	ND			ND[e]
Cg14	Seep	pos. rod	-	ND			ND[e]
Cg15	Seep	neg. rod	+	*Ps. picketii*			*Pseud. fluorescens* E 0.681
Cg16	Seep	neg. rod	+	No I.D.			*Pseud. corrugata* 0.530
Cg17	Seep	neg. rod	+	No I.D.			No I.D.
Cg18	Seep	pos. rod	-	ND			*Micrococcus diversus* 0.703
Cg20	Seep	pos. rod	-	ND			ND[e]
Cg21	Seep	neg. rod	-	*Sphingomonas paucimobilis*			*Sphingobacterium mizutaii* 0.683
Hg1	Hillside	neg. rod	-	No I.D.			No I.D.
Hg2	Hillside	neg. rod	-	No I.D.			No I.D. (*Ps. glathei* 0.472)
Hg3	Hillside	neg. rod	-	No I.D.			*Pseudomonas glathei* 0.575
Hg4	Hillside	neg. rod	-	No I.D.			*Ps. glathei* 0.507
Hg5	Hillside	neg. rod	-	No I.D.			*Ps. glathei* 0.523
Hg6	Hillside	neg. rod	-	*Pseud.* sp.			No I.D.
Hg7	Hillside	neg. coccus	-	No I.D.			ND
Hg8	Hillside	neg. rod	-	*Pseud.* sp.			*Burkholderia gladioli* 0.54
Hg9	Hillside	neg. rod	-	*Pseud.* sp.			*Burk.* sp. (*gladioli* 0.45)
Hg10	Hillside	neg. rod	-	*Pseud.* sp.			*Burk. gladioli* 0.596
Hg11	Hillside	neg. rod	-	No I.D.			No I.D.
Hg12	Hillside	neg. rod	-	*Pseud.* sp.			No I.D. (Ps. gladioli 0.34)
Hg13	Hillside	neg. rod	-	No I.D.			*Burk.* sp. (*gladioli* 0.40)
Hg14	Hillside	neg. rod	-	No I.D.			No I.D.
Hg15	Hillside	neg. rod	-	No I.D.			No I.D. (*Ps. fluorescens* A 0.36)
Hg16	Hillside	neg. rod	-	No I.D.			No I.D. (*Ps. glathei* 0.21)
Hg17	Hillside	neg. rod	-	*Pseud.* sp.			*Burk. gladioli* 0.52
Hg18	Hillside	neg. rod	+	No I.D.			*Ps. glathei* 0.502
Hg19	Hillside	neg. rod	-	*Pasteurella haemolytica*			No I.D. (*Ps. glathei* 0.486)
HG20	Hillside	neg. rod	-	No I.D.			*Xanthomonas maltophilia* 0.752
HG21	Hillside	neg. rod	-	No I.D.			No I.D.
HG22	Hillside	neg. rod	-	*Pseud.* sp.			*Bu. gladioli* 0.562

[a] Names in parentheses indicate the species within the identified genus with the highest % id value.

[b] An estimate of how closely the isolates profile corresponds to the proposed taxon relative to all other taxa in the API-NFT data base. This value ranges between 0 and 100. An "acceptable" identification, as defined by the manufacturer, is one with a % id $\geq$ 80.0. Values in parentheses are those for the closest species given for an identified genus.

[c] Measure of the isolates proximity to the most typical profile in each taxon. The value of T varies between 0 and 1. Values in parentheses are those for the closest species given for an identified genus.

[d] ND = not determined

[e] These Gram-positive isolates are similar, based on colony morphology and color, cell morphology, and DNA fingerprinting, to Cg3 and Cg18, both identified by BIOLOG as *Micrococcus diversus*.

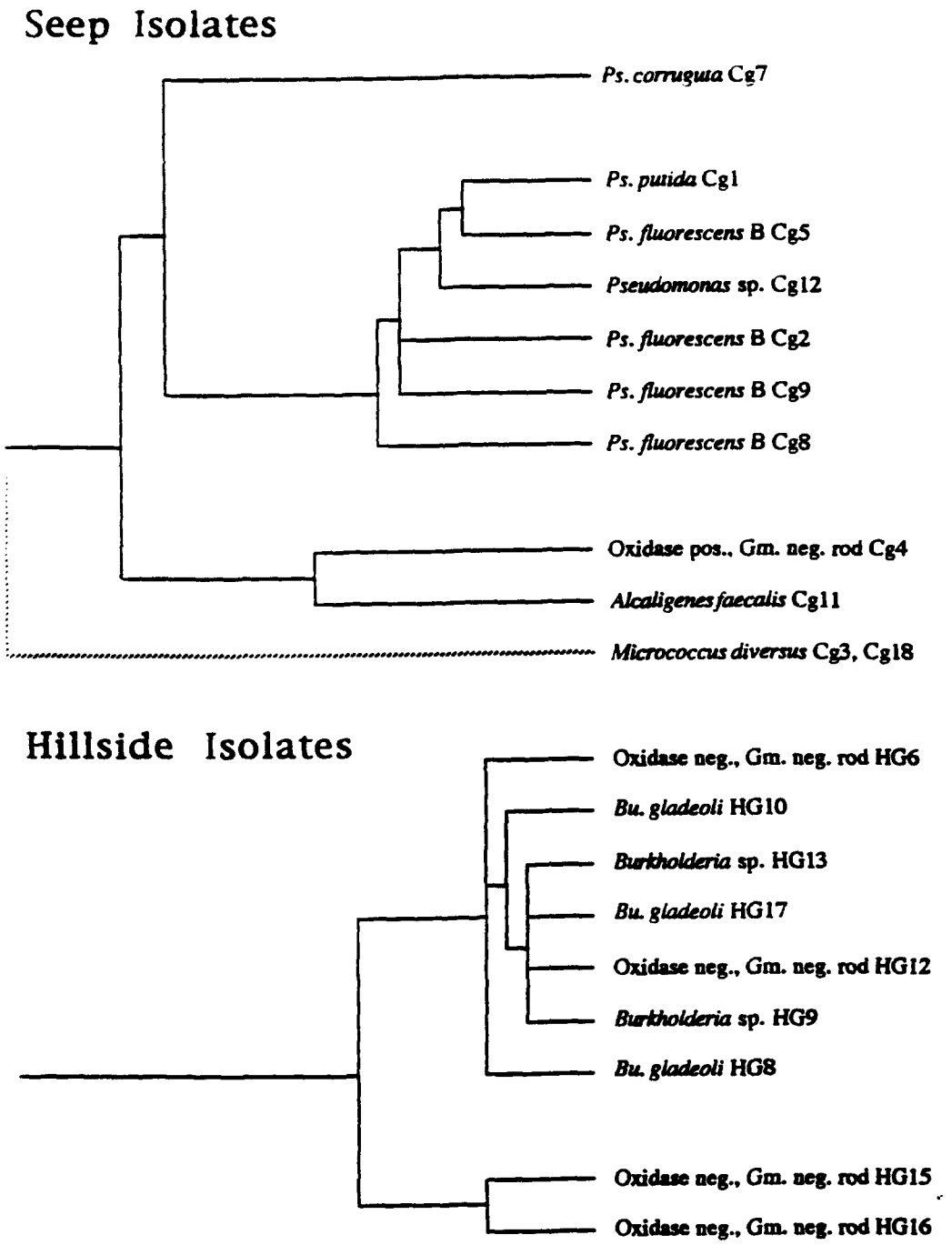

Figure 26. Cluster analysis based on BIOLOG carbon source utilization patterns of selected naphthalene mineralization isolates.

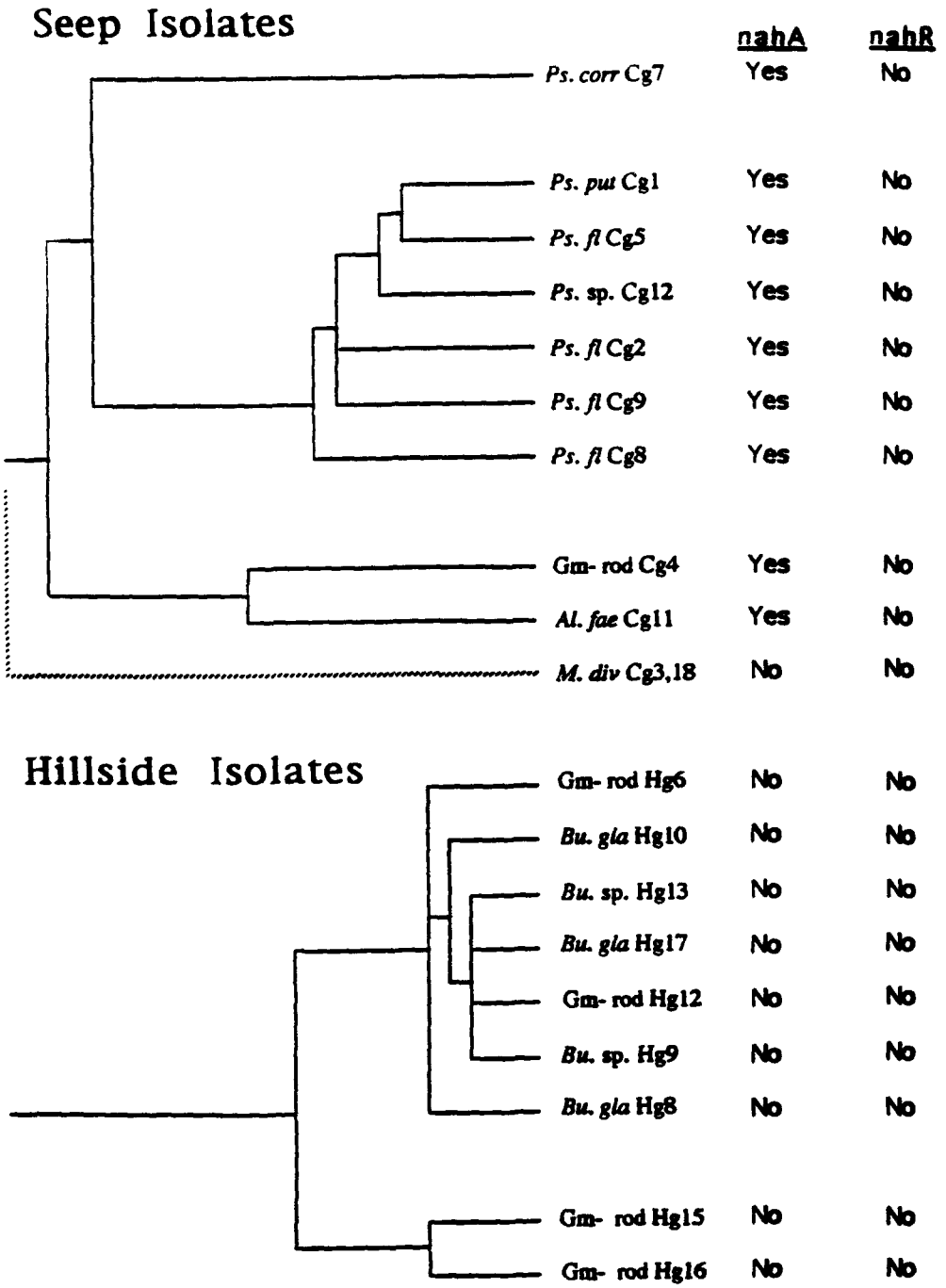

Figure 27. Distribution of PCR Detectable *nah* genes among naphthalene-mineralizing isolates.

to be important in laboratory naphthalene metabolizing strains, we are exploring the genetic diversity of naphthalene metabolism that is carried out by a real-world microbial community in the field. Although the polymerase chain reaction (PCR) assay used here has certain limitations (i.e., specificities and idiosyncrasies implicit in primer design and amplification conditions, especially stringency), the contrast between genetic characteristics among the bacteria isolated in this study and between these and the laboratory type strain (*P. putida* Pp67, supplying the base pair sequence information) is striking.

Additional experiments exploring the details of how and why genetic catabolic variation has developed at our study site are in progress.

Conclusions

1. Naphthalene-degrading isolates from the contaminated sediment and from the uncontaminated soil represent two very different taxonomic groups, with different patterns of *nah* gene amplifiability.
2. The presence at the site of homologs to the type PpG7 naphthalene dioxygenase gene *nah*Ac suggests that genes closely related by descent to those of the type strain are involved in naphthalene catabolism in actual field situations.
3. Lack of amplifiability of genes in site-derived isolates using PpG7 PCR primers and conditions has been found. This may be caused by a variety of mechanisms including gene sequence divergence or functional convergence of alternate pathways. Experiments exploring these issues are in progress.

2.5. Mobility of PAHs and Bacteria Capable of PAH Metabolism in Sediments

In order to investigate the degree to which organic contaminants and bacteria capable of contaminant metabolism are mobile in the seep area of the study site, a field experiment was implemented. The overall approach was to insert sorbents into the organic rich sediments and periodically remove them for both microbiological and chemical analysis. The sorbents utilized were clean, sterilized polyurethane foam enclosed in fiberglass mesh (for PAH sorption) and 2-g portions of sterilized site-derived sand, also enclosed in fiberglass packets (for sorption of microorganisms). The experimental design placed 20 of each of these sorbents into the sediments to depths of 3 cm. The sorbents were inserted (in 4x5 arrays) along the flow path of water as it exits the seep area and flowed toward the nearby surface stream and distant river. Periodically, at approximately weekly intervals, the four replicates of the sorbents were removed from the sediment using a randomized design and subjected to extraction and chemical analysis (foam sorbents) or

microbiological analysis (sand sorbent). To minimize sampling artifacts, within minutes after removal from sediments, the foam sorbents were aseptically unwrapped, freed of excess water (by squeezing), placed into Teflon sealed vials and an extractant (1:1 acetone hexanes) was added. Later in the laboratory, GC/mass spectral analysis was completed.

After removal from the site, the sand sorbents were processed by aseptically transferring the sand packets to sterile plastic bags and then keeping the samples at 4°C. Dilution and plating of the microorganisms colonizing the sand occurred back in the laboratory within 24 h using a general heterotrophic media (PTYG) and PAH-specific media (mineral salts + naphthalene and mineral salts + phenanthrene).

Results of the field mobility experiments are presented in Figs. 28 and 29. both naphthalene and phenanthrene-utilizing bacteria were mobile and reached peak titers of 10^4 and $10^{3.3}$, respectively within 11 days of sorbent insertion into the sediment (Fig. 28). These data unquestionably reveal that heterotrophs, including PAH-metabolizing bacteria, are present in the water and sediment at the field site. Initially sterile sand was colonized on Day 0 after a 1 min immersion in the sediment. Furthermore, the numbers of heterotrophs occupying the sandy sorbent changed with time of sampling.

This experiment was designed to assess "colonization" i.e., the dynamic changes in components of the microbial community over time. These changes were brought about by the transport of microorganisms into the two grams of sorbent and/or by the subsequent growth of the microorganisms. But the sampling methodology could not easily distinguish these two mechanisms of change from one another. The population shifts shown in Fig. 28 may be due to a major colonization event at t = 0 followed by subsequent transport and growth related population changes.

The degree to which population shifts in Fig. 28 were caused simply by instantaneous equilibration with the surrounding sediment versus growth and accrual on the initially sterile sorbent can be assessed by comparing the numbers of bacteria retrieved from the sterile sorbent to the number of bacteria retrieved from the site sediments at each sampling time. In panels A and B of Fig. 28, the initial bacterial numbers in the sediment were between 1.6 and 2.5 log units higher in the sediment than in the briefly immersed sand sorbent. Then, over the next two sampling periods, numbers increase asymmetrically in the sand sorbents towards those in the surrounding sediment. Thus, for general heterotrophs (panel A) and naphthalene metabolizing bacteria (panel B) the data suggest that gradual colonization and changes occurred on the initially sterile

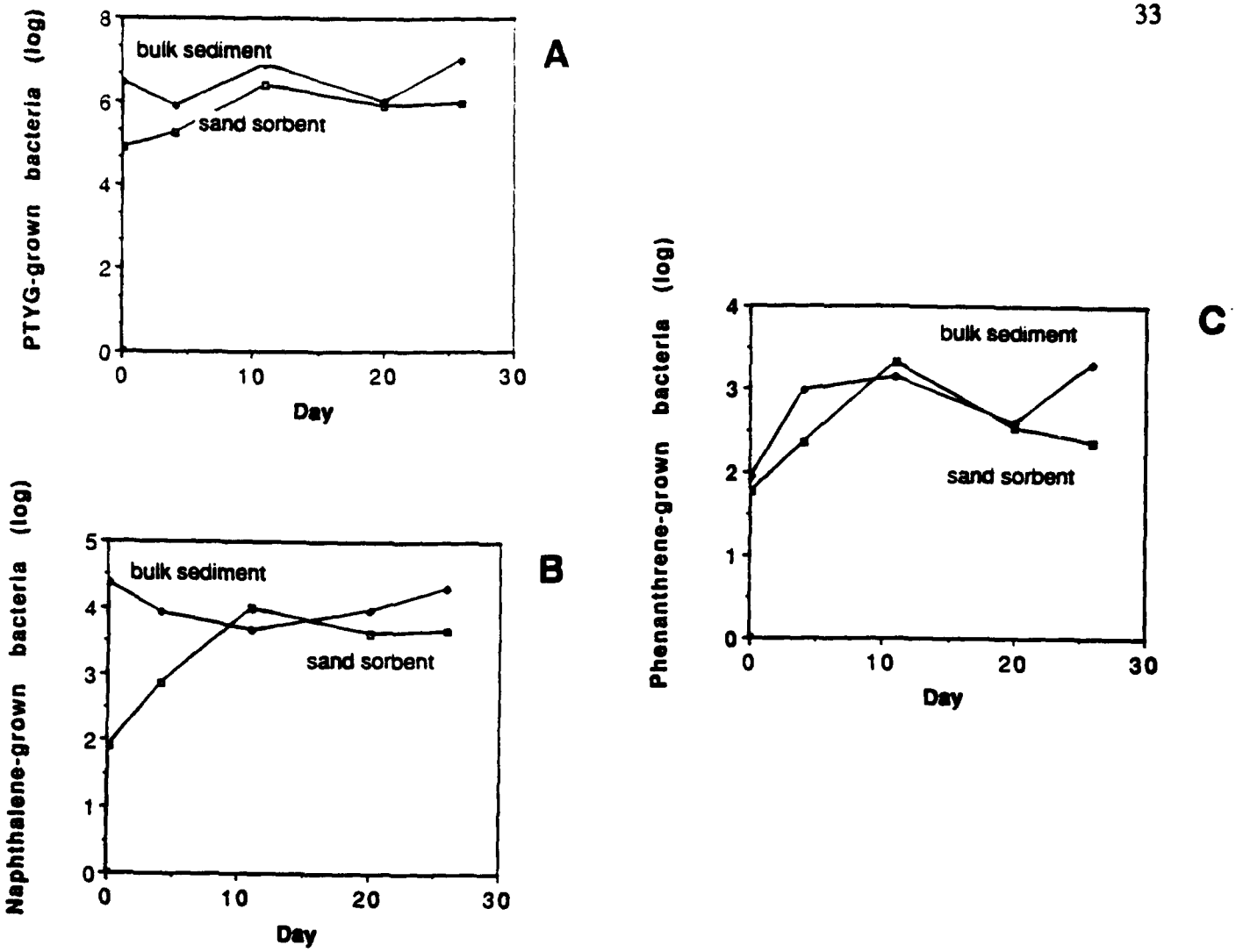

Figure 28. Numbers of naphthalene-, phenanthrene-, and PTYG (a general heterotrophic medium)-degrading bacteria retrieved at various times from initially sterile sand sorbents inserted into the seep study area Fall 1993. Each type of bacteria was enumerated after aseptic serial dilution and plating onto agar media containing each respective carbon source.

Panel A shows bacterial numbers from bulk sediment (single unreplicated sample) and sand sorbent (4 replicate samples) growth on PTYG media.

Panel B shows bacterial numbers from bulk sediment (single unreplicated sample) and sand sorbent (4 replicate samples) growth on naphthalene.

Panel C shows bacterial numbers from bulk sediment (single unreplicated sample) and sand sorbent (4 replicate samples) growth on phenanthrene.

sorbent. By contrast, for phenanthrene metabolizing bacteria (whose numbers were 3 and 1 order of magnitude lower than those from PTYG and naphthalene media, respectively), there was no distinguishable difference between bacterial numbers in bulk sediment versus those retrieved from the sand sorbent (panel C). Thus, the data shown in panel C do not argue for dynamic successional population shifts within an initially sterile microhabitat. Instead of accrual and growth of phenanthrene-utilizing bacteria on the sorbent; the population shifts seem to appear to simply reflect act simply an instantaneous equilibration with the ambient water and sediment.

Fig. 29 displays the results of chemical analyses performed on the foam sorbents installed in the seep area and later analyzed by gas chromatographs/mass spectrometry. The eight compounds shown were selected because of their relatively high abundance and consistent appearance in chromatograms. The initially clean polyurethane foam plugs were immersed in saturated sediments, then after periods ranging from 5 minutes to 23 days analyses were performed. Interpreting the data in Fig. 29 requires the same vigilance used to interpret data in Fig. 28.

Processes reflected in the contaminant retrieval data in Fig. 29 include sorption/desorption reactions, aqueous phase transport, filtration of colloids into the foam sorbent, and microbial metabolism of the compounds sampled by the foam. With the exception of data describing 3 Nitro-1,2-dicarboxynaphthalene, all compounds were initially at very low concentrations. This implies that, unlike the microbial colonization experiments described above, the foam sorbents did not instantaneously equilibrate with their surroundings. In other words after initial insertion into the site sediments, the subsequent changes in concentration of the compounds reflected *in situ* sorption, transport and biodegradation dynamics. Seven of the eight compounds did not simply accrue in the foam sorbent (for uncertain reasons the exception was 3,-nitro-1,2-dicarboxybenzene). Instead, a maximum concentration was found on day 15 followed by a slow decline in concentration by day 23. The reason for the decline in concentration was uncertain. However, both leaching of the materials, microbial metabolism, and/or a shift in the relative rates of loss versus accrual mechanisms may be responsible.

Summary of Mobility Experiments

The impetus for inserting sorbents for microbial cells and organic contaminants into the field site sediments was to ascertain how dynamic these key components are at the site. The ambient concentrations of contaminants and cells in the sediments reflect a balance of mechanisms that add (via transport, sorption, cell growth) and remove (via transport, desorption, biodegradation, cell death) the chemicals. Although the interactions

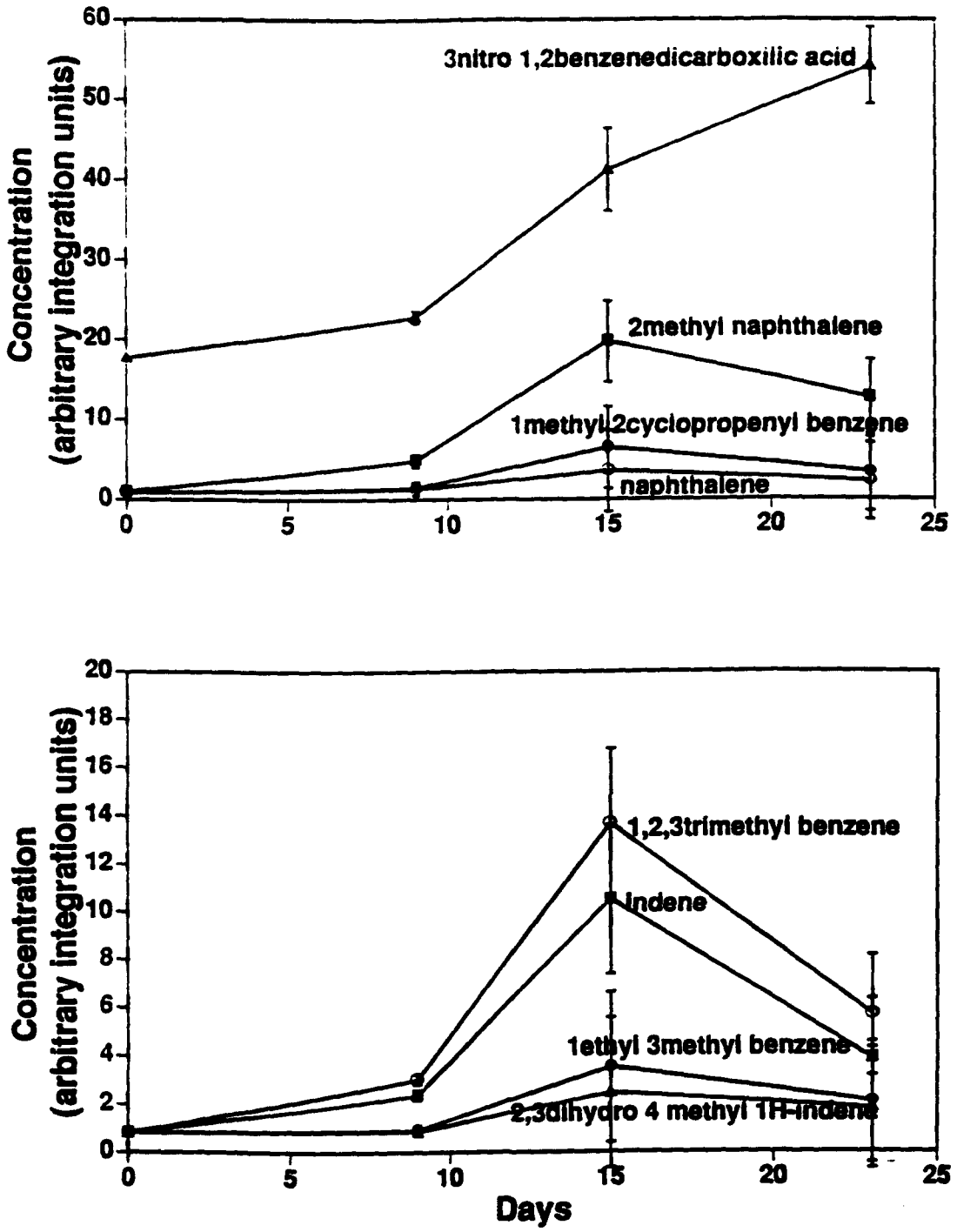

Figure 29. Concentrations of coal tar-derived compounds retrieved from polyurethane foam sorbents inserted into the seep study area Fall 1993. Each point represents the average of 3 or 4 replicate samples. Compounds are analyzed by gas chromatography/mass spectrometry. Authentic standards were not available for all compounds therefore, absolute concentrations were not ascertained. Instead, the arbitrary units of chromatogram peak areas are displayed along the vertical axes.
Top = concentrations of naphthalene, 2 methylnaphthalene, 1 methyl, 2 cyclopronyl, benzene, and 3 nitro-1,2dicarboxybenzene
Bottom = Concentrations of indene, 1,2,3-trimethylbenzene, 1, ethyl-2, methylbenzene, and 2,3-dihydro-4 methyl-1H indene.

of these processes may be complex and difficult to discern, the procedures used to implement this initial inquiry were straight forward and insightful. Principle conclusions are:

1. Contaminant metabolizing heterotrophic microorganisms indigenous to the field site are mobile, capable of colonizing initially sterile sand, and vary in abundance over time.
2. Naphthalene metabolizing bacteria occur at a density of 10^4/g, compared to 10^3 for phenanthrene degraders. These abundances of the respective bacteria are consistent with the relatively high solubility and concentration of naphthalene at the site.
3. Eight naphthalene, indene, or benzene derivatives were consistently identified by GC/MS analysis following extraction from foam sorbents inserted into the seep area at the study site.
4. The concentrations of seven of the eight compounds mentioned under point #3, increased during a 15 day period and the gradually declined. The reason(s) for this pattern is uncertain, but may reflect a combination of contaminant mobility, solubility, water flow events, and biodegradation.

2.6. Development of Methods for Extracting and Analyzing DNA From Sediments

The appended manuscript entitled "Quantitative Cell Lysis of Indigenous Microorganisms and Rapid Extraction of Microbial DNA From Sediments" has been accepted and will appear in the May 1994 issue of Applied and Environmental Microbiology.

3.0 FUTURE PLANS

During the next funding period for this project, we will continue to build on existing results and test hypotheses aimed at explaining the persistence of PAHs at our study site.

- Experiments examining the behavior of *Pseudomonas paucimobilis* RSP1 and its metabolism of phenanthrene will continue. Microscopic examination of sediment-cell substrate interactions may be explored.
- Experiments testing the Sorption/Bioavailability hypothesis will continue.
- Experiments exploring the genetic diversity of naphthalene metabolism among site-derived bacteria will continue.
- During the Summer of 1994, additional field experiments will be implemented.

APPENDIX I

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Quantitative Cell Lysis of Indigenous Microorganisms and Rapid Extraction of Microbial DNA from Sediment

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This study reports improvements in two of the key steps, lysis of indigenous cells and DNA purification, required for achieving a rapid nonselective protocol for extracting nucleic acids directly from sodium dodecyl sulfate (SDS)-treated organically rich sediments. Incorporation of bead-mill homogenization into the DNA extraction procedure doubled the densitometrically determined DNA yield (11.8 μg of DNA · g of cellular dry weight^{-1}) relative to incorporation of three cycles of freezing and thawing (5.2 μg of DNA · g of cellular dry weight^{-1}). The improved DNA extraction efficiency was attributed to increased cell lysis, measured by viable counts of sediment microorganisms which showed that 2 and 8%, respectively, survived the bead-mill homogenization and freeze-thaw procedures. Corresponding measurements of suspensions of viable *Bacillus* endospores demonstrated that 2 and 94% of the initial number survived. Conventional, laser scanning epifluorescence phase-contrast, and differential interference-contrast microscopy revealed that small coccoid bacterial cells (1.2 to 0.3 μm long) were left intact after combined SDS and bead-mill homogenization of sediment samples. Estimates of the residual fraction of the fluorescently stained cell numbers indicated that 6% (2.2 × 10^8 cells · g of cellular dry weight^{-1}) of the original population (3.8 × 10^9 cells · g of cellular dry weight^{-1}) remained after SDS and bead-mill homogenization. Thus, lysis of total cells was less efficient than that of cells which could be cultured. The extracted DNA was used to successfully amplify *nahR*, the regulatory gene for naphthalene catabolism in *Pseudomonas putida* G7, by PCR. By scaling down the mass of soil extracted to 0.5 g and by using gel purification and SpinBind DNA purification cartridges, the time required to extract DNA from whole sediment samples was reduced to 2 h.

Microbial ecologists, systematicists, and population geneticists have become increasingly interested in methods for complete, unbiased isolation of DNA (7, 9, 12, 16, 29, 30) and RNA (6, 8, 11, 19, 34, 36) from soils and sediments because such procedures promise to make the genomes of uncultured indigenous microorganisms available for molecular analysis. The ideal (2, 35, 36) is to circumvent the biases implicit in culture-based procedures by directly accessing the genes of naturally occurring microbial communities. But achieving this ideal requires overcoming a variety of interferences that diminish the quality, yield, and diversity of extracted nucleic acids. These interferences raise questions about the completeness of nucleic acid extraction, and about the representativeness of results based on the procedures.

The popular direct lysis approach to DNA extraction and purification (24) may be dissected into the following conceptual steps: (i) washing the material to remove soluble components that may impair manipulation of subsequently isolated DNA; (ii) disruption of cells in the material to release DNA or RNA from the cells; (iii) separation of the DNA or RNA from solids; and (iv) isolation and purification of the released DNA or RNA so that it can be used in various molecular procedures (i.e., PCR, digestion by restriction enzymes, hybridization reactions, or sequencing). A variety of methods integrating most or all of these steps have been published (7, 12, 20, 22, 28, 29, 31), yet, no study has demonstrated that the DNA or RNA was extracted from soil or sediment completely. Nor have criteria for complete extraction of DNA or RNA from native soil and sediment communities been established.

Procedures for lysis of microbial cells in soils and sediments have relied on one or more of the following treatments: lysozyme, heat, proteinase K, sodium dodecyl sulfate (SDS), achromopeptidase, hot phenol, guanidine thiocyanate, pronase, acetone, Sarkosyl, EDTA, freeze-thaw cycles, freeze-boil cycles, sonication, bead-mill homogenization, microwave heating, and mortar mill grinding. Ogram et al. (20) reported that a combination of SDS (incubated at 70°C) and bead-mill homogenization achieved a 90% lysis efficiency for cells native to marine and freshwater sediments, as determined by microscopic counts. Tsai and Olson (31) reported that an EDTA-lysozyme treatment followed by three freeze-thaw cycles reduced microscopic counts of cells added to sediment and subsoil samples by 95%. Similarly, Picard et al. (22) reported that three sonication microwave-thermal shock cycles achieved complete lysis of *Streptomyces* spores. More recently, Erb and Wagner-Döbler (7), using microscopic counts of two bacterial strains added to sterile sediments, concluded that six SDS, freeze-thaw treatments led to 99% lysis efficiency. While all of these reports were based on microscopic observations, descriptions of surviving-cell size distribution and morphology have yet to be presented. Furthermore, general criteria for lysis efficiency of microorganisms native to sediments have yet to be established. In this regard, several investigators (7, 22, 31) have made the questionable assumption that test microorganisms added to sediments were valid surrogates for native cells.

The rationale for the use of a lytic procedure is clear: complete disruption of cellular structure and release of nucleic acids is the objective. A goal of our research was to better understand the effectiveness of cell lysis procedures by determining their effects on the diverse assemblage of cells in native

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microbial communities. In this investigation, we compared the effects of two of the most widely used physical lysis procedures, cycles of freezing and thawing and bead-mill homogenization, on DNA yield and viable-cell plate counts. Total counts and microscopic observations of acridine orange-stained samples were also used as criteria for lysis effectiveness. Finally, we simplified the protocol for extraction and purification of DNA from the sediment.

MATERIALS AND METHODS

Sediment samples. Sediment samples were obtained aseptically from a coal tar-contaminated site near South Glens Falls, New York. Sample characteristics and other details of the site have been described previously (12, 17, 18). Approximately 35 years ago, coal tar was buried in a single depositional event, and since that time, groundwater flow has distributed soluble coal tar constituents in a narrow contaminant plume through sandy subsurface sediments. The contaminated water, which contains naphthalene and phenanthrene, emerges in an organic matter-rich seep area at the foot of a hill slope, 400 m down-gradient from the original coal tar deposit. The methods described here primarily utilized the seep sediment, in which organic matter content was approximately 20% and the water content was approximately 50% (12). Other sandier sediments (approximately 1% organic matter and 20% water) were also used in this study: these subsurface sediments, designated "source," "upgradient," and "downgradient," were obtained from boreholes at the field site along a midline transect of the plume (17, 18). In samples from source, upgradient, and downgradient locations, the concentrations of polycyclic aromatic compounds, especially naphthalene and phenanthrene, gradually diminished. Storage of seep and subsurface samples (in presterilized screw-cap glass jars) was at 4°C for periods up to 1 and 3 years, respectively. Any changes in microbial populations that may have occurred during storage were immaterial for the purposes of this investigation.

Cell lysis. The following general lysis protocol was used in all experiments. Equal weights (either 0.25 or 0.5 g) of wet sediment and phosphate buffer (100 mM, pH 8 [23]) were added sequentially to 2-ml screw-cap polypropylene microcentrifuge tubes (Laboratory Products Sales, Inc., Rochester, N.Y.) containing 2.5 g of 0.1-mm-diameter zirconium silicon dioxide beads (BioSpec Products, Bartlesville, Okla.) previously sterilized by autoclaving for 50 min at 120°C and 15 lb/in^2. Next, 0.25 ml of a 10% SDS solution (SDS-Tris-NaCl: 100 mM NaCl–500 mM Tris, pH 8–10% SDS) was added; the final concentration of SDS was approximately 4%. Each tube was shaken at high speed for 5 or 10 min in a bead-mill homogenizing unit (BioSpec Mini-Bead Beater). The selection of bead size and the proportion of beads to cell suspension were determined by following guidelines for disrupting bacterial cells provided by the manufacturer. The tubes were removed from the bead-mill and centrifuged for 3 min at 12,000 × *g*.

To compare the lysis efficiency of the bead-mill homogenization and freeze-thaw procedures, the sediment was mixed by adding 3 g of sediment to 3 ml of phosphate buffer in a 15-ml plastic centrifuge tube and mixing for 2 min on vortex mixer; 0.5 ml of the mixed suspension (equivalent to 0.25 g of sediment) was immediately distributed to the 2-ml microcentrifuge tubes with and without prior addition of 0.1-mm beads as described above. A 0.5-ml suspension of *Bacillus* endospores in the phosphate buffer was also added to microcentrifuge tubes with and without beads. Endospores were harvested from a culture of *Bacillus subtilis* CU 1065 (Section of Microbiology, Cornell University) by culturing the bacterium on 5% PTYG agar medium [4, 5] and allowing extensive (approximately 40 days) desiccation to occur at 22°C. The spores were harvested by flooding the plate with the phosphate buffer. Microscopic examination showed that 100% of the *Bacillus* cells in the suspension had sporulated. Each tube received 0.25 ml of the 10% SDS–Tris–NaCl solution. The SDS-containing suspensions of the spores or sediment were then subjected to two different lysis procedures. In the freeze-thaw procedure, samples were rapidly frozen by immersion in liquid nitrogen (2 min) and then thawed in a 65°C water bath (5 min); this freeze-thaw cycle was carried out three times. The bead-mill homogenization procedure was carried out for 5 min as described above, with or without beads added. In this case, lysis efficiency was evaluated by triplicate viable-cell plate counts on 5% PTYG agar medium and microscopically as described below. The results were confirmed in three separate experiments, though data from only one are reported here.

Microscopic evaluation of cell lysis. Intact sediment samples or samples treated with SDS and subjected to the lysis procedures were stained with 0.01% acridine orange and examined with either a Zeiss Standard 18 microscope under phase-contrast and epifluorescence viewing or a Zeiss laser scanning microscope (model LSM-10) equipped for fluorescence, phase, and differential interference contrast imaging under 488-nm light from an argon laser. The LSM-10 is configured such that a single field of view can be examined by conventional transmitted and epifluorescence illumination or by comparable laser-scanning illumination. Both microscopes are fitted with ×100 oil immersion objective lenses with numerical apertures of 1.3 or 1.4. An acridine orange direct count (AODC) agar-smear procedure (5, 10) was used to assess the extent of lysis of the endospores and enumerate the total number of cells in the sediment. The computerized imaging and analysis systems of the LSM-10 were used to document the size distribution of microbial cells surviving the various lytic procedures. In enumerating cells in the sediment prior to implementing lysis procedures, the average count and standard deviation were computed from duplicate smears prepared from three independent subsamples of the sediment as described previously (5). In lysis experiments, the same general procedure was followed, except that only one smear from each sample was examined. In one instance, the number of surviving cells was estimated from a wet mount of a known volume of sample under a 22-mm^2 coverslip.

DNA purification. The supernatant from the lysis treatment (150 to 250 μl) was mixed 2:5 with a volume of 7.5 M ammonium acetate, and a precipitate was allowed to form for 5 min at 4°C. Then, the tube was spun for 3 min at 12,000 × *g* and 150 μl of the supernatant was concentrated and partially purified with a SpinBind DNA extraction cartridge (FMC BioProducts, Rockland, Maine). In a SpinBind cartridge, the DNA binds to a microporous silica membrane in the presence of chaotropic salts; after washing, the DNA can be eluted with water. The units were used according to the manufacturer's instructions, except that an EDTA-free ethanol wash buffer was employed and the DNA was eluted with 30 μl of warm (60°C) deionized water. The eluted DNA was loaded onto a 1% agarose gel containing 0.5% ethidium bromide and subjected to electrophoresis (4 V/cm) for 20 min in TAE buffer according to a standard protocol (3). The resulting DNA bands were cut out of the gel and purified with a SpinBind cartridge according to the manufacturer's instructions for extraction from an agarose gel.

Quantification of DNA. The concentration of DNA after the final purification step was measured by densitometry as fol-

TABLE 1. *Effect of freeze-thaw treatment and bead-mill homogenization on survival of culturable sediment bacteria and Bacillus endospores*

Sample	Treatment[a]	CFU (±SD) · gdw^{-1}[b]	% Survival	Viable/total cell ratio (%)[c]
Sediment	None	$1.0 (\pm 0.2) \cdot 10^7$	100	0.3
	SDS + freeze-thaw	$7.8 (\pm 1.5) \cdot 10^5$	8	0.02
	SDS + 5-min bead-mill[d]	$1.5 (\pm 0.2) \cdot 10^5$	2	0.004
Endospores	None	$1.8 (\pm 0.1) \cdot 10^8$	100	ND[e]
	SDS + freeze-thaw	$1.7 (\pm 0.3) \cdot 10^8$	94	ND
	SDS + 5-min bead-mill	$3.5 (\pm 0.5) \cdot 10^6$	2	ND

[a] See Materials and Methods.
[b] gdw, gram of cellular dry weight.
[c] Also see Table 2; AODC of untreated sample = $3.8 (\pm 0.3) \times 10^9$ cells · gdw^{-1}.
[d] When beads were omitted from the homogenization procedure, the postlysis CFU count was $6.5 (\pm 1.0) \cdot 10^6$ (65% survival).
[e] ND, not determined.

lows: 3.0 μl of each sample and 4, 2, 1, and 0.5 μl of Lambda DNA standards (Promega, Madison, Wis.) cut with *Hin*dIII (New England Biolabs, Beverly, Mass.) were electrophoresed on a 1% agarose gel in TAE running buffer as described above. The gel was photographed under a Spectroline 302-nm UV transilluminator (model TR-302) as previously described (37). A negative image of the gel was produced with a Polaroid MP4 Land camera using Polaroid type 55 film. Bands on the negative were scanned with a laser densitometer (Helena Laboratories, Houston, Tex.), and the DNA was quantified by interpolation from a calibration curve prepared from the densities of Lambda-*Hin*dIII-cut DNA standards.

PCR amplification of extracted DNA. The suitability of the isolated DNA to undergo enzymatic amplification reactions was tested by a nested PCR protocol (15, 27), using primers for *nahR*, the regulatory gene in the naphthalene catabolism gene cluster encoded on the NAH7 plasmid of *Pseudomonas putida* (39). The outer primer sequences were 5'AACTGCGTGAC CTGGATTTAA3' and 5'CGCCGCCGGCTCGGCTGGTG T3', corresponding to nucleotides 152 to 172 and 1244 to 1224 (39) of the *nahR* gene. The inner primer sequences were 5'G CCGCGCATCTGGCCGAGCCCGTCACTTCGG3' and 5'C TGGAGGATGTGGCCAACGGCGGCGAAGTGC3', corresponding to nucleotides 343 to 373 and 1200 to 1170 of the gene. The final product was 828 bp long. Reagents and conditions for carrying out the PCR were as previously described (12), except that the inner and outer reactions were prepared under "hot-start" conditions, with the deoxynucleoside triphosphates added after the tubes were heated to 80°C. The outer reaction mixture included 2 μl of SpinBind-purified sample and was cycled 1 time at 95°C for 5 min; 5 times at 94°C for 2 min, 65°C for 1 min, and 72°C for 1 min; 25 times at 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s; and 1 time at 72°C for 5 min. For the inner amplification which followed, 5 μl of solution produced from the outer reaction mixture was used as template. Tubes were cycled 30 times at 95°C for 30 s and 70°C for 1 min and 1 time at 72°C for 5 min. The PCR products were detected by agarose gel electrophoresis in 1% agarose gels as described above. *P. putida* G7, used as a positive control in the PCR assay, was originally obtained from G. S. Sayler (University of Tennessee) and was grown at 30°C in 5% PTYG as previously described (12). Negative controls in the PCR assay were done with reagent only (i.e., no added DNA) and a blank derived from a peripheral piece of the DNA purification gel that was taken through purification and amplification procedures.

RESULTS

One indication of the effectiveness of cell lysis procedures is cell viability. Therefore, we measured the change in viable bacteria (CFU) before and after bead-mill homogenization and freeze-thawing as an indicator of the extent of lysis. The data in Table 1 show that the bead-mill homogenization (2% survival) was more effective than freeze-thawing (8% survival) in reducing CFU of SDS-treated sediment bacteria. The CFU data only accounted for 0.3 to 0.004% of the total microscopic counts (Table 1); therefore, the survival rate of bacteria after the two cell lysis procedures was also tested with endospores of *B. subtilis*. Because of their resistance to physical disruption, endospores can serve as a model for other resistant microbial structures. The ineffectiveness of the freeze-thaw procedure in reducing the viability of a suspension of *B. subtilis* endospores was striking (94% survival [Table 1]) relative to the bead-mill homogenization, after which 2% of endospores remained viable. Lack of viability corresponded to the physical disruption of cell walls after bead-mill homogenization (Fig. 1). Phase-contrast microscopy showed that the phase-dense, refractile spores were completely ruptured after bead-mill homogenization (Fig. 1). The usual bright green fluorescence characteristic of DNA stained with acridine orange was missing in the ruptured spores and, therefore, had been released into the solution.

To further confirm that the reduced viability (Table 1) and ruptured cells (Fig. 1) were indicative of an extracellular release of DNA, we measured the yield of DNA from 0.5 g of sediment extracted and purified by several variations of the above lysis procedures: 5-min bead-mill homogenization as described above; three freeze-thaw cycles; or 5 min of bead-mill homogenization followed by three freeze-thaw cycles and then another 5 min of bead-mill homogenization. Initial qualitative examination of the yields from these three lysis methods was accomplished via 1% agarose gel electrophoresis (data not shown). On the basis of the fluorescence of intercalated ethidium bromide, there was no clear visual difference between the two treatments that utilized bead-mill homogenization. This suggested that bead-mill homogenization, alone, was as effective as a combination of freeze-thaw treatment and bead-mill homogenization in releasing DNA. In contrast, the intensity of the DNA band resulting from the freeze-thaw treatment alone was dimmest, thus corroborating the lower lysis efficiency of this treatment relative to bead-mill homogenization (Table 1). The DNA from all three lysis preparations was then concentrated with a SpinBind cartridge, employed in this study to improve DNA recovery over ethanol precipitation-DNA resuspension procedures used earlier (12). A portion of each

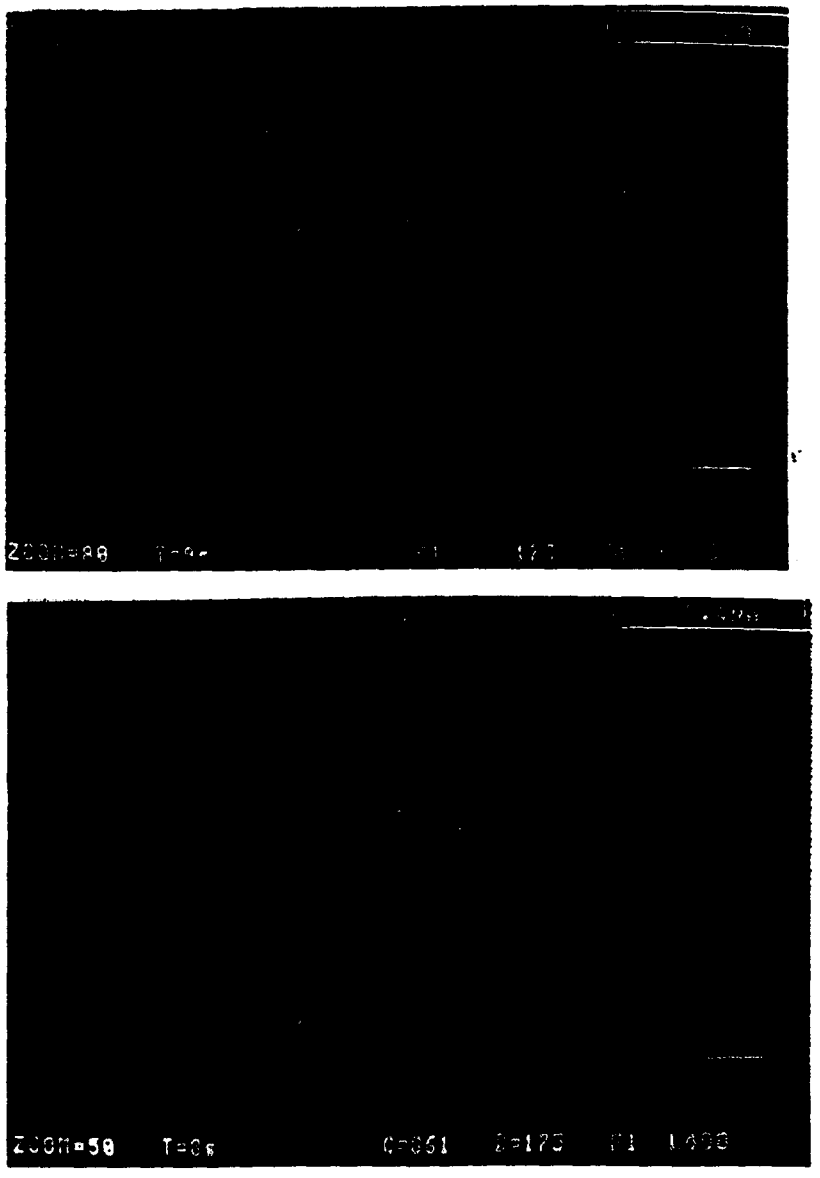

FIG. 1. Phase-contrast micrographs of *B. subtilis* spores before (A) and after (B) bead-mill homogenization. Dimensions in the upper righthand corner of each micrograph denote the distance between the crosses marking the ends of cells. Bars, 5 (A) and 10 (B) μm.

concentrated DNA preparation was next electrophoretically purified on a 1% agarose gel. Each DNA band was excised from the gel, processed a second time with the SpinBind cartridge, and visualized on an agarose gel, and then a negative image of each band was scanned with a laser densitometer to quantify the DNA. Yields from the bead-mill homogenization (alone), freeze-thaw treatment (alone), and the two lysis treatments combined were 11.8, 5.2, and 11.0 mg of DNA · g^{-1} of sediment, respectively. Thus, freeze-thaw treatment, alone, released one-half as much DNA as from the two bead-mill homogenization treatments, whose DNA yields were virtually indistinguishable.

Microscopic examination of the sediment provided an additional means of assessing the response of native microorganisms to lytic procedures. Because the data in Table 1 and the above DNA yields clearly demonstrated that freeze-thawing was a less effective cell lysis method than bead-mill homogenization procedures, only the latter and several variations (aimed at discerning the role of SDS in the procedure) were investigated. Table 2 reports the total bacterial numbers (AODC), approximate size distribution, and morphological diversity of microorganisms in sediments before and after SDS treatment, bead-mill homogenization, or both treatments. Prior to lysis, the sediment sample contained a rich and varied collection of both eukaryotic and prokaryotic cells which spanned a wide range of cell sizes (Table 2). In general, the various size fractions diminished as the severity of lytic procedures increased. The key observation shown in Table 2 is that

TABLE 2. Effect of bead-mill homogenization and SDS treatment on total bacterial numbers, bacterial size distribution, and diversity in organically rich sediment

Treatment[a]	AODC (±SD) · gdw^{-1}[b]	AODC remaining (%)	Approx size range[c] (μm)	Morphological diversity[c]
Untreated	3.8 (±0.3) · 10^{10}[d]	100	>10–0.3	I
5-min bead-mill	1.9 (±0.5) · 10^{9}	50	2.0–0.3	III
10-min bead-mill	1.0 (±0.2) · 10^{9}[e]	26	2.0–0.3	III
SDS	4.9 (±0.4) · 10^{8}	13	5.0–0.3	II
SDS + 10-min bead-mill	2.2 (±1.6) · 10^{8}[f]	6	1.2–0.3	III

[a] See Materials and Methods; note that the SDS reagent was included in the bead-mill homogenization procedure reported in Table 1.
[b] gdw, gram of cellular dry weight.
[c] Size range and morphological diversity of fluorescent cells in at least 15 microscopic fields observed during AODC counting by conventional and laser scanning epifluorescence phase-contrast and differential interference-contrast microscopy. *I*, large and small filaments, rods, cocci, sarcina-like clusters of microcolonies. *II*, sarcina-like clusters and small coccoid cells only. *III*, small coccoid cells only.
[d] A factor of 380 greater than the untreated sample CFU described in Table 1.
[e] The AODC of this sample was estimated by determining the number of green fluorescent cells per ×1,000 field of 10 μl of a 1:8 diluted sample containing acridine orange spread under a 22-mm^2 coverslip.
[f] A factor of 1,467 greater than the SDS- and bead-mill-treated CFU described in Table 1.

approximately 6% [2.2 (±1.6) × 10^8 cells · g of cellular dry weight^{-1}] of the bacteria, mostly small coccoid cells in the sediment, were unaffected even by bead-mill homogenization in the presence of SDS. The small cells which resisted lysis were observed by laser scanning epifluorescence microscopy (Fig. 2). It is important to note that the epifluorescence images are produced electronically in black and white by using a green analyzer filter and photomultiplier detector. Therefore, the degree of brightness of an object in these images was directly related to green fluorescence. It is also important to note that the ratio of viable to total counts (CFU/AODC) of the original sample was 0.3% before treatment (Table 1). After treatment with SDS and 10 min of bead-mill homogenization, the CFU/AODC ratio was 0.07% (compare the data in Table 1 and Table 2, footnote *e*). Thus, the net effect of these combined treatments was to lyse the larger cells that were more likely than the small cells to grow on the plate count medium.

PCR amplification of sediment-derived DNA. In addition to examining the efficacy of cell lysis procedures, this study also pursued the goal of achieving a rapid overall procedure for extracting and purifying DNA from sediment. By scaling down the total amount of sediment processed, from 1 (12) to 0.25 or 0.5 g, we were able to perform all of the above procedures in microcentrifuge tubes. This, in combination with utilization of SpinBind units, shortened the total processing time, from crude sediment to purified DNA, to approximately 2 h.

Many reports have shown that soil and sediments contain humic or other substances that may remain associated with extracted DNA, thus preventing its subsequent analysis (13, 29, 33). To determine if the DNA yielded from sediment samples was pure enough to allow subsequent molecular analysis, we performed a variety of tests. The first was designed to ascertain the effectiveness of gel electrophoresis in DNA purification. A 1-μl volume containing 45 *P. putida* G7 cells (determined by plate counts) was added to the PCR mixture along with 2 μl of sediment-derived DNA that had twice been passed through the SpinBind cartridges, with and without gel electrophoretic purification in between (the particular sediment subsample used here lacked amplifiable *nahR*). After completion of the nested PCR procedure on both preparations, *nahR* was amplified from *P. putida* G7 cells only with the electrophoretic purification (data not shown). Thus, we confirmed our previous results (12) indicating that the sediment contained PCR-inhibitory substances whose removal required a gel-electrophoretic purification step.

Prior work has shown that sediment samples from a variety of locations in our coal tar-contaminated field site contain genes homologous to *nahA* (12) and *nahR* (27). Using the DNA isolation and purification protocol described here, we repeatedly examined the quality of the DNA so obtained. Figure 3 shows the PCR products that resulted from four different sediment samples from our study site. Because PCR is sensitive both to concentrations of inhibitory substances and to the concentration of target DNA sequences, we amplified directly after the final elution from the SpinBind unit (Fig. 3, lanes 1, 3, 5, and 7) and after a 10-fold dilution (Fig. 3, lanes 2, 4, 6, and 8). The DNAs extracted from seep sediment (used to develop the protocols described in this study [Fig. 3, lanes 1 and 2]) and source sediment (Fig. 3, lanes 7 and 8) were susceptible to PCR amplification of the *nahR* gene, regardless of dilution. However, DNA preparations from the other sediment samples displayed differing responses to dilution. *nahR* was not amplified from the diluted upgradient sediment DNA (Fig. 3, lane 4)—possibly indicating a low titer of target DNA. In contrast, the DNA preparation from the downgradient sediment yielded a relatively weak amplification band in the undiluted sample Fig. 3, lane 5)—possibly indicating that the electrophoresis and SpinBind purification steps failed to completely remove substances inhibitory to the PCR. As an additional negative control treatment in the experiment whose results are shown in Fig. 3, a piece of the purification gel from outside the DNA bands was carried through the PCR procedure and failed to yield the amplified product (data not shown). When additional subsamples of the sediments used (Fig. 3) were repeatedly carried through the DNA extraction, purification, and PCR procedures, amplification of the *nahR* genes was not always consistent. This inconsistency was noted previously in the amplification of *nahAc* from the upgradient and downgradient samples (11). The reason for this variability is uncertain, but the variation may have been caused by heterogeneity inherent in the physical, chemical, and microbiological characteristics of field site-derived sediments.

DISCUSSION

This report has articulated the role of cell lysis as the first in a series of procedures required for achieving efficient, nonselective access to the genes in naturally occurring sediment microbial communities. But, perhaps more importantly, we have presented criteria for evaluating the effectiveness of the lysis step. These criteria were loss of cell viability, total DNA yield, and microscopic examination of sediment-derived cells

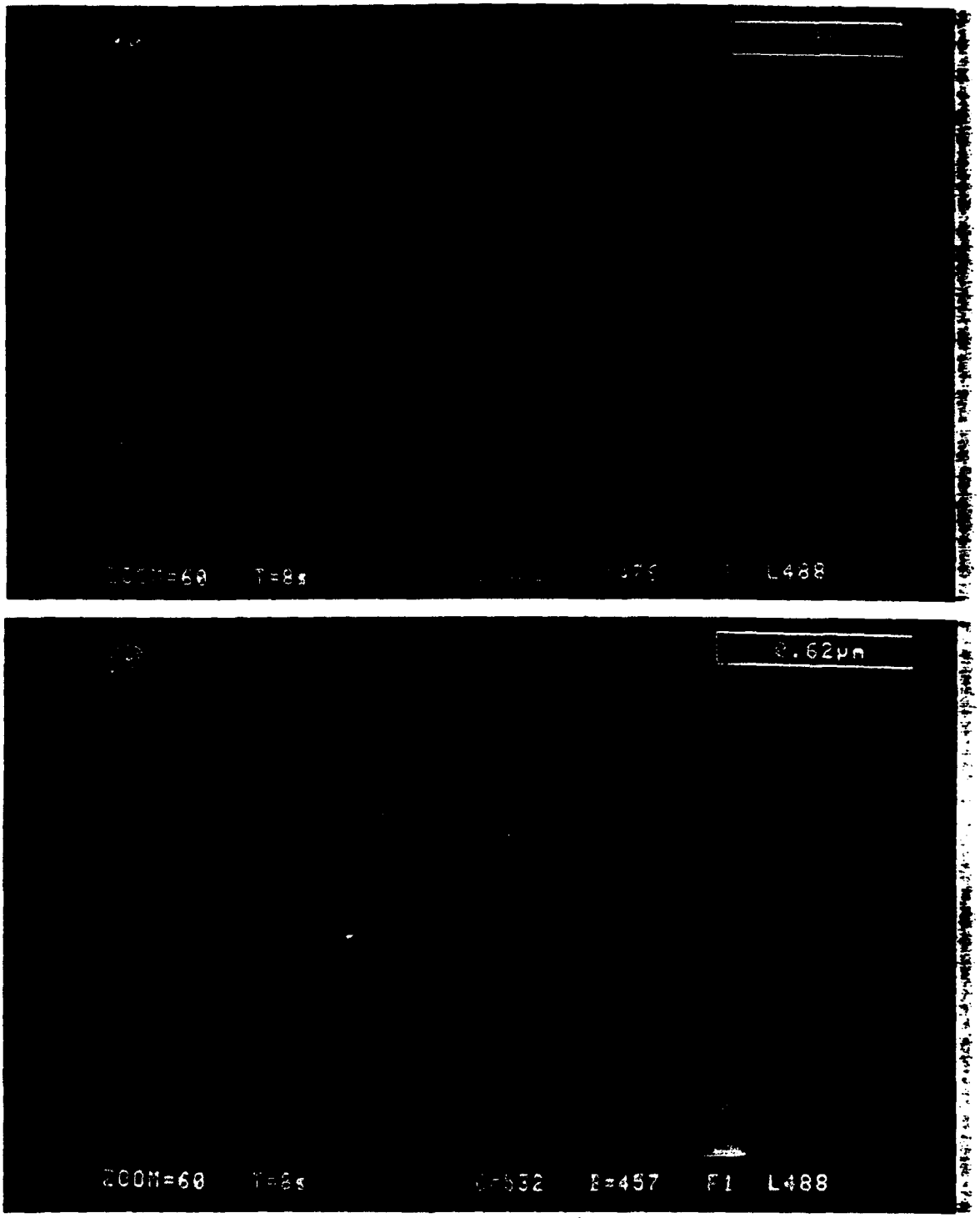

FIG. 2. Laser scanning epifluorescence micrographs of acridine orange-stained native cells in sediment before (A) and after (B) bead-mill homogenization. Note the presence of both large and small cells in the center of the prelysis micrograph. Only very small cells remained after lysis. Dimensions in the upper righthand corner of each micrograph denote the distance between the crosses adjacent to cells or particles.

for total direct counts and morphological diversity changes. By all four criteria, bead-mill homogenization was shown to be more efficient in lysing cells than freeze-thawing. Furthermore, the quality of the DNA subsequently extracted from the sediment was verified by PCR amplification of a native naphthalene catabolic gene.

Precedent has been set for using the behavior of an indicator microorganism, often seeded into sediments prior to determin-

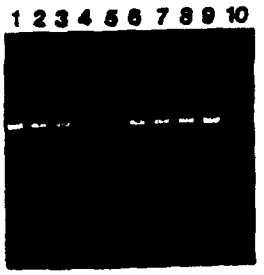

FIG. 3. Amplification of *nahR* from four sediment samples with nested primer PCR. For lanes 1, 3, 5, and 7, a 2-μl concentrated sample was used as a template for PCR amplification; and for lanes 2, 4, 6, and 8, a 2-μl 1:10 dilution of the same samples was used. Lanes: 1 and 2, seep sediment; 3 and 4, upgradient sediment; 5 and 6, downgradient sediment; 7 and 8, source sediment; 9, positive control (part of a *P. putida* G7 colony added to the PCR mixture); 10, PCR negative control (reagent only).

ing the efficiency of lysis or DNA recovery, as a basis for extrapolating to the behavior of indigenous cells (7, 22, 31). Similarly, in this study, viable counts of *Bacillus* endospores and native sediment bacteria were examined as a means for testing cell lysis procedures. A consistent proportion of surviving cells (2%) (Table 1) shared by the two very different microbial assemblages (total sediment microorganisms and *Bacillus* endospores) provided initial encouragement that DNA released from sediment by bead-mill homogenization would be completely representative of the sediment microbial community. However, the total viable counts derived from the sediment represented only 0.3% (prelysis) (Table 1) and 0.07% (postlysis) (Table 2, footnote *e*) of the total microscopic count. This total microscopic count necessarily included unknown proportions of nonviable but intact cells and both cultured and uncultured viable cells. Thus, an astonishingly large component of the sediment microbial community studied here was characterized only according to microscopically discernible traits such as cell size and morphology (Table 2). The SDS, bead-mill homogenization treatment disrupted indigenous cells in a biased manner by leaving the smallest size fraction (1.2 to 0.3 μm long) (Table 2; Fig. 2) intact. Until this resistant portion of the sediment microbial community can be lysed (perhaps by using smaller beads and additional chemical lytic agents), the ideal of accessing all of the indigenous genes will be thwarted. Furthermore, it is clearly unwise to use added indicator microorganisms, or even viable indigenous cells as a basis for drawing inferences about the susceptibility of the uncultured microbial community to cell lysis procedures.

Despite the fact that SDS, bead-mill homogenization failed to disrupt small cells native to the sediment, it is appropriate to use the data presented here to estimate total sediment DNA and the overall efficiency of the extraction procedure. If we presume that prokaryotes were the predominant reservoir of sediment DNA and that each of the $3.8 \cdot 10^9$ prokaryotic cells · g of cellular dry weight^{-1} contained a single stationary-phase genome weighing $5 \cdot 10^{-15}$ g (based on data for *Escherichia coli* [38]), then 1 g (dry weight) of the sediment contained 19 μg of DNA. This value agrees reasonably well with the total sediment DNA estimated by Ogram et al. (20) (27 μg · g of cellular dry weight^{-1}) and with the ranges of total soil DNA (20 to 50 μg · g of cellular dry weight^{-1}) reported by Picard et al. (22), Selenska and Klingmüller (26), and Steffan and et al. (28). Factors contributing to variability in total DNA estimates include those imposed by different extraction methodologies, as well as microbiological idiosyncrasies of particular samples stemming from physiological influences such as soil or sediment type, climate, and the content of water, oxygen, and organic matter, etc. The DNA yielded when SDS, bead-mill homogenization was combined with the extraction protocol described here (11.8 μg · g of cellular dry weight^{-1}) represents 62% of the 19 μg of total theoretical DNA. Many of the assumptions contributing to this efficiency figure are uncertain; nonetheless, this estimated yield is reasonably high. It is perhaps remarkably high in light of the fact that much of the DNA from the 1.2- to 0.3-μm-long cell fraction was not released (Table 2) and that the steps subsequent to cell lysis (especially separation of the DNA from sediment particles) were not carefully scrutinized. Only after each step has been thoroughly examined and optimized can DNA extraction biases be reduced and efficiency be increased. It should be noted that even if an extraction efficiency of 99.9% were achieved, with 10^9 cells per g this would still leave 10^6 organisms per g unsampled. Thus, even when the lysis efficiency is relatively high, minor members of the community may remain intact and, consequently, their DNA may escape detection. Although there is no clear solution to this dilemma, we feel that continued striving towards the combination of unbiased genome sampling and enhanced sensitivity afforded by PCR may partially mitigate such detection limit problems.

PCR detection of genes native to sediment requires that the ratio of target sequence be high relative to accompanying sediment-derived materials that may inhibit the denaturation, annealing, and DNA synthesis stages of PCR (29, 32, 33). In this regard, optimal sensitivity for amplifying native genes can only be achieved by separating the DNA from inhibitory substances. Recently, Abbaszadegan et al. (1) have shown that Sephadex G-100 and Chelex 100 resins successfully removed PCR-inhibitory substances from groundwater concentrates. Perhaps ironically, nontarget DNA itself has also recently been shown to mask the PCR amplification of target sequences in low abundance (29). This study has confirmed that purification of DNA extracts is required for successful PCR amplification of indigenous genes (*nahR*). But even in such purportedly pure DNA preparations, lack of amplification in undiluted DNA extract (Fig. 3) suggested that inhibitory substances still remained in the mixture. The need to dilute DNA extracts prior to PCR amplification has been reported earlier for electrophoretic purification of DNA extracted from environmental samples (7, 22, 33), and it is the simultaneous dilution of the target sequence that may ultimately limit the sensitivity of the method.

Scale and its equivalent, sample size, are other issues to be considered in performing and interpreting experiments examining molecular characteristics of naturally occurring microbial communities. The small-scale (0.5 g) processing of sediment reported here substantially diminished the logistical and time constraints on DNA extraction. But facile processing of small samples raises questions about how accurately such small samples represent microbial communities as they occur in the landscape. Not enough is known about the chemical, physical, and microbiological spatial heterogeneity of soils (21) and sediments to allow data from 0.5-g samples to be the basis for extrapolation to larger (i.e., kilogram) or very large (i.e., landscape) scales. Moreover, the amplifiability of genes present in 0.5-g samples undoubtedly reflects the variable spatial distribution of both the target DNA sequence and sediment-derived substances that inhibit PCR (see the discussion above). Because these determinants for successful gene amplification may vary independently, interpreting the results of such assays may prove challenging.

Recently, Erb and Wagner (7) used DNA extraction and PCR amplification techniques to obtain a polychlorinated biphenyl catabolic gene directly from a German freshwater sediment. A comparison of restriction digests failed to detect any divergence between a sediment-derived *bphABC* gene fragment and that of the type strain, *Pseudomonas* sp. strain LB400 (7). In contrast, by hybridizing DNA extracted from soils with a variety of gene probes Holben et al. (14) have recently demonstrated that the genetic basis in soil microbial communities for 2,4-dichlorophenoxyacetic acid catabolism was broader than that of plasmid pJP4. Similarly, we reported significant restriction fragment length polymorphism relative to *P. putida* G7 in the *nahAc* genes in DNA extracted from the same coal tar-contaminated field site examined here (12). In order to learn more about the distribution of related naphthalene catabolic gene sequences, procedures in this study utilized a different, nested set of oligonucleotide primers, specifically designed to amplify an 828-bp fragment of the *nahR* gene (27, 39). *nahR* is a member of the *lysR* family of regulatory genes that are widely distributed among gram-negative bacteria (25). Detection of *nahR* in the DNA extracted from the sediment provides two types of information. First, amplification of this gene allowed the quality of sediment-derived DNA to be evaluated. Because PCR amplification was possible, we concluded that the rapid extraction and purification procedures developed in this investigation were successful. But, perhaps more interestingly, detecting *nahR* in DNA extracted from this field study site lends additional momentum to ecological inquiries which utilize DNA sequence information from pure culture-derived functional genes to explore gene distribution and variation in nature.

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